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STRUCTURE-FUNCTION RELATIONSHIP OF HYDROPHIIDAE POSTSYNAPTIC NEUROTOXINS

FINAL REPORT

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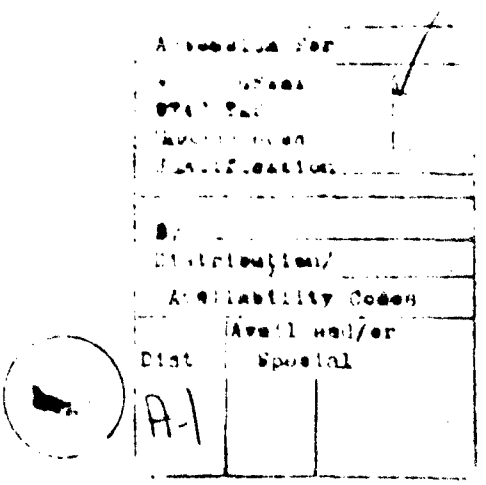
13. Abstract (continued)

function studies of gila toxin from a gila monster venom concluded that gila toxin is an arginine esterase with kallikrein-like activity causing lethality and gyration in mice. However, it is not a postsynaptic neurotoxin.



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13. ABSTRACT *(Maximum 200 words)*

Lapemis toxin, from a Hydrophiidae sea snake venom (Lapemis hardwickii), binds tightly and specifically to the nicotinic acetylcholine receptor (AChR) inhibiting neumuscular transmission and results in muscular paralysis. In order to study the structure-function relationship of Lapemis toxin structural loop domains were synthesized. The domain peptide were found to be non-toxic at 8 mg/kg dosag.. (i.v., mice) or 114 times the known LD50 of Lapemis toxin. Binding studies with Torpedo californica acetylcholine receptor and [125-I] radiolabelled toxin and peptides indicated that Lapemis toxin and peptide B1 bound with equilibrium dissociation constants of 2 nM and 40 nM, respectively. The other peptides had no detectable binding. Chemical modification study of which of the three arginines, located in the central loop, are involved in the neurotoxin-AChR demonstrated that Arg-31 and Arg-34 residues are involved in toxin-AChR interaction. The central loop B with intact disulfide bond of the native lapemis toxin appears to play a dominate role in the toxin's binding ability to the receptor. Characterization of the 9 kD and the 13.5 kD proteins fractionated from Lapemis hardwickii venom have been identified from partial sequence data as parvalbumin and phospholipase A2, respectively. Structure-

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List of Abbreviations

AChR, acetylcholine receptor;
LTX, lapemis toxin;
α-Bgtx, α-bungarotoxin;
HPLC, high performance liquid chromatography;
DTT, dithiothreitol;
DTNB, 5,5' dithio bis (2-nitro)benzoic acid;
TFA, trifluoroacetic acid;
CA, carboxyamidomethylated;
BCA, bicinchoninic acid;
ODS, octaldodecylsilane;
SDS, sodium dodecylsulfate (lauryl sulfate);
PAGE, polyacrylamide gel electrophoresis;
PIP, piperidine;
DMF, N,N-dimethylformamide;
HOBt, 1-hydroxybenzotriazole;
OPFP, pentafluorophenyl ester;
tBU, tert-butyl;
Fmoc, 9-fluorenylmethoxycarbonyl;
Mtr, 4-methoxy -2,3,6 trimethylbenzenesulfonyl;
Boc, tert-butyloxycarbonyl;
OtBu, tert-butyl ester;
DCM, dichloromethane;
ODhbt, 1-oxo-2-hydroxy-dihydrobenzo-triazine ester;
PTH, phenylthiohydantoin;
ATZ-aa, anilinothiazolinone amino acid derivative
K_D, Equilibrium dissociation constant
RP, Reverse Phase

Section 1.

Introduction

Snakes and snake venoms have been the source of much interest over the years. Of the about 2,340 known species of snakes, approximately 420 species are venomous (Dowling & Duellman, 1978). Of these 420 species approximately 240 species are the fixed-front fang (proteroglyphous) snakes which are extremely toxic.

Snake venoms contain many components ranging from enzymes to small peptides such as neurotoxins. The majority of the components or factors are responsible for diverse physiological and pathological actions. Among these components are postsynaptic neurotoxins and factors that increase capillary permeability (Tu, 1977; Lee, 1979; Endo & Tamiya, 1987; Shier & Mebs, 1990; Tu, 1991).

Snake venoms have been found to contain at least four different categories of neurotoxic components: 1) Post-synaptic neurotoxins (Lee, 1972; Tu, 1977; Dufton & Hider, 1983; Mebs & Hucho, 1990); 2) Presynaptic neurotoxins (or β-neurotoxins) (Hawgood & Bon, 1991); 3) Acetylcholinesterase inhibitors (Harvey et al., 1984); and 4) Potassium channel blockers (Halliwell et al., 1986).

The Hydrophiidae family is one of the five families of poisonous snakes and the only true sea snakes. The other four families of poisonous snakes are the Elapidae (cobras, mambas, and kraits), Viperidae (vipers), Crotalidae (pit vipers) and Colubridae (rear-fanged snakes) inhabiting warm climate regions of the land. Sea snakes inhabit the coastal waters in the tropical and subtropical regions of the Pacific and Indian Oceans. They are not found in the Atlantic Ocean, Arctic Ocean, Red Sea nor cooler regions of the Pacific and Indian Oceans (Tu, 1988).

Sea snakes are classified as the family Hydrophiidae which is divided into two subfamilies of 15 total genera and 50 species. The two subfamilies are Hydrophiinae and Laticaudinae (Table 1-1). The Hydrophiinae subfamily is divided into twelve genera with 36 known species. The Laticaudinae subfamily is divided into three genera with 13 known species (Smith, 1926; McDowell, 1972; Voris, 1977; Tu, 1988). The Hydrophiinae lack distinct ventral scales dictating their entirely aquatic lifecycle. The Laticaudinae, however have ventral scales, allowing them to crawl onto beaches.

Sea snake venom is a mixture of different proteins and nonprotein components synthesized in the venom gland and stored until the snake bites. Table 1-2 lists the protein components that have been detected in the venom of various sea snake venoms. Only phospholipase A_2, phosphomonoesterase, and postsynaptic α-neurotoxins have been isolated and further characterized from Hydrophiidae venoms. Table 1-3 tabulates the protein components found in different snake venoms, which have been shown not to occur in sea snake venom or have not been studied. Arginine esterases, proteases and L-amino acid oxidase were studied but not detected in sea snake venoms. From this table it is evident that many future studies and projects are possible reguarding sea snake venoms.

Sea snake venom contains potent postsynaptic neurotoxins. The dosage at which 50 percent of mice die (LD_{50}) for sea snake venom are reported as low as 0.044 mg/Kg mouse body weight for *Aipysurus duboisii* venom injected subcutaneous (Minton, 1983). This high toxicity is due mainly to the neurotoxin. Yields of venom from sea snakes are relatively small compared to most land snakes with ranges from 0.6 to 19.0 mg per snake in up to nine drops, depending on the species. One drop (0.05 ml) of sea snake venom is said to contain enough toxin to kill three adults. Because a venom contains highly toxic postsynaptic neurotoxins, venom as a whole exhibits high toxicity. The potent neurotoxins such as Lapemis toxin can cause muscle paralysis and respiratory failure of a victim which may lead to morbidity or death (Reid, 1979). The pharmacological action of these neurotoxins are similar to that of curare (tubocurarine), an alkaloid arrow poison used by South American Indians, thus these postsynaptic neurotoxins are the so called curaremimetic neurotoxins. Sea snake neurotoxin strongly binds to the acetylcholine receptor at the neuromuscular junction blocking the nerve transmission to the diaphragm, and subsequent respiratory failure is the main cause of the high toxicity of sea snake venom and the

neurotoxin. Table 1-4 lists many of the known sea snake neurotoxins along with their LD_{50} values and route of injection. The purified neurotoxins have reported LD_{50} values ranging from 0.04 mg/kg to 0.31 mg/kg in mice.

To date, all isolated sea snake neurotoxin are postsynaptic neurotoxins (some times termed α-neurotoxins) with the neuromuscular nicotinic acetylcholine receptor as the target. Sea snake neurotoxins are predominately of the short chain (type I) postsynaptic polypeptides with 60-62 amino acid residues with four disulfide bonds. These toxins range in molecular weights from 6,500-7,000 and isoelectric points of 8.5-10.0. For comparison, several long-chain (type II) toxins have also been isolated and sequenced. The long chain neurotoxins contain 71-74 amino acid residues and five disulfide bonds. A couple of neurotoxins have structures between type I and II and have been termed hybrid neurotoxins; they contain four disulfide bonds but have many other features similar to type II neurotoxins (Tu, 1988).

Postsynaptic neurotoxins are very compact and stable molecules due to their small size and relatively high di-sulfide bond content capable of withstanding boiling and yet retain structure and toxicity (Tu et al., 1976).

The amino acid sequences of 31 sea snake neurotoxins have been reported (Tu, 1988; Mebs & Hucho, 1990) and listed in Table 1-5. A high degree of structure homology exists when comparing sea snake neurotoxins with that of Elapidae neurotoxins with many invariant residues and many highly conserved residues (Low, 1979; Dufton & Hider, 1983).

The secondary structure and tertiary structure of the short chain neurotoxins have been shown by Raman, CD, NMR and X-ray diffraction to consist of β-sheet and β-turns with no α-helix. Table 1-6 tabulates the toxins that have had their secondary structures determined and by which method or methods.

Lapemis toxin is one of these known short chain neurotoxins with a molecular weight of 6,800, pI of 9.6, four disulfide bonds, 60 amino acid residues and a reported LD_{50} of 0.06 mg/Kg (i.v., mice) (Tu & Hong, 1971). The primary structure of the toxin was determined by Fox and coworkers (1977) (Table 1-5) and the secondary structure was determined showing the toxin consisted of β-sheet and β-turns with no α-helix (Table 1-6). The tertiary structure of Lapemis toxin is depicted in Figure 1-1 based on the X-ray diffraction results of a similar sea snake toxin (*Laticauda semifaciata* toxin b) (Tsernoglou & Petsko, 1976). From this figure, note the compact nature and the three prominent structural loops termed A, B, and C starting from the N-terminal of the molecule. The four disulfide bonds of the neurotoxin are clustered in one area and there is the protruding loop B.

The sequences of the subunits of the neuromuscular nicotinic acetylcholine receptor have been deduced from the cDNA sequences of the four subunits known to make up the pentamer receptor. These subunits are designated as $\alpha_2\beta\ \delta$ for several species including the Torpedo californica receptor (Noda et al., 1983, Claudio et al., 1983).

The neuromuscular nicotinic acetylcholine receptor is perhaps the best studied membrane receptor (Galzi et al., 1991; McCarthy et al., 1986; Conti-Tronconi & Raftery, 1982; and Changeux, 1981). Figure 1-2 portrays a model of the molecular structure of the AChR in the postsynaptic cell membrane. Two ACh Receptors are linked by a disulfide bridge between the two δ-subunits. For each ACh Receptor there are five subunits, two homologous α-subunits, a β-subunit, a -subunit and the δ-subunit that are arranged around the ion channel (Endo & Tamiya, 1987).

Studies of sea snake neurotoxins and their interaction with acetylcholine receptors (AChR) aimed at determining structure-function relationships of these toxins with respect to their target receptor have been intense. There are many related review articles on this subject (For examples: Tu, 1988; Dufton and Hider, 1983; Lee, 1979; Atassi, 1991).

In order to understand Lapemis toxin's action, the current study has been designed to elucidate the binding region(s) of the neurotoxin on the AChR. Specifically, the study asks the questions:

1. Which part or parts of the neurotoxin bind(s) to the receptor?
2. What are the relative binding abilities of the selected synthesized regions of the toxin to the receptor?

Answers to these questions will allow further effective design of experiments aimed at finding which regions and amino acids of the toxin are important in the direct binding to the receptor.

In order to localize the binding domains of Lapemis toxin and α-neurotoxins toward the acetylcholine receptor four main strategies have been or should prove useful:

1. Enzymatic/Chemical cleavage of the toxin and evaluation of the relative binding of the purified fragments.
2. Chemical modification of selected neurotoxin amino acids for which a known specific modification method is available.
3. The use of peptide synthesis to produce peptides of the same or modified sequence of α-neurotoxins such as Lapemis toxin to deduce each region's involvement in the binding.
4. The use of molecular biology cloning and monoclonal antibody techniques.

The use of these combined strategies will enable the direct amino acid interactions of the toxin with the receptor to be determined and give related structure-function information.

The use of chemical modification has helped to provide important insight on the structure and function relationship of snake neurotoxins. Some amino acid residues were chemically modified and the effects of these modifications on toxicity or acetylcholine receptor binding ability of the postsynaptic neurotoxins were investigated (eg. Sato & Tamiya, 1970; Seto, et al., 1970; Tu & Hong, 1971; Tu, 1988). Chemical modification of sea snake neurotoxins is summarized in Table 1-7. Of the modified residues, the sole Tryptophan residue, some Arginine residues, some Lysine residues, and perhaps the sole Tyrosine residue have a role in the toxicity and binding ability of the neurotoxins. The disulfide bonds when modified also demonstrated a structural importance for the toxicity of the neurotoxins.

The receptor binding of acetylcholine, specific antibodies, and α-neurotoxins have been extensively studied. Neurotoxins which exhibit high-affinity binding to the nicotinic AChR are prominent components of nearly all elapid and hydrophiid venoms (Lee, 1979; Tu, 1990). Typically these are small basic proteins with molecular weights of approximately 6000-8000 Daltons; all of the α-toxins have considerable sequence homology (Chiapinnelli, 1985). Computer-assisted modeling of α-toxin binding to AChR suggests that a "tryptophan cleft" on the toxin may entrap trp187 on the AChR subunit (Low and Cornfield, 1987). These toxins bind to the synaptic surface of each α-subunit of the AChR (Kubalek et al., 1987). Interestingly, a thyroid-derived protein, thymopoietin, has been shown to be a potent inhibitor of α-bungarotoxin binding to neuronal nicotinic AChR; the authors suggest that thymopoietin may be an endogenous ligand for α-bungarotoxin receptors in neuronal tissue (Quik et al., 1989). Several viruses have been shown to utilize receptors for endogenous ligands to target and to infect host cells (e.g., Eppstein et al., 1985). Rabies virus glycoprotein has considerable amino acid sequence similarity to several snake venom neurotoxins (Lentz et al., 1984). The finding that the rabies virus glycoprotein binds to the α-subunit of the nicotinic AChR and inhibits binding of α-bungarotoxin further supports the probability of this site being "intended for" a native ligand (Bracci et al., 1988). These data raise the possibility that α-toxins (such as Lapemis neurotoxin) may recognize binding site(s) on muscle AChR that are also actually designed for endogenous peptides/proteins (perhaps a modulator binding site) in muscle endplate tissue.

It has been suggested that the AChR α-subunits each contain five α-toxin binding regions (Mulac-Jericevic and Atassi, 1987). The α-neurotoxins α-bungarotoxin (from Bungarus multicinctus; Formosan Krait) and cobratoxin (from Naja naja; Indian cobra) have been localized to five main regions on the α-subunit of the receptor of the Torpedo receptor but only three regions of the human receptor. At this time it is not known if these regions bind different neurotoxin molecules or are different faces interacting with the toxin (Mulac-Jericevic and Atassi, 1987a, 1987b; Mulac-Jericevic et al., 1988).

Recent studies investigating binding of α-toxins to AChR have utilized synthetic peptide fragments of the α-subunit of the AChR and snake venom neurotoxins, most notably α-bungarotoxin. A peptide consisting of residues 183-

200 of the AChR α-subunit (containing the disulfide bridge of cys192 and cys193) has been shown to bind α-bungarotoxin, indicating that this sequence may also participate in cholinergic binding (Takamori et al., 1988). When the cys192-193 disulfide is reduced, binding of α-bungarotoxin decreases by 85% (Gotti et al., 1987). Antibodies against the AChR α-subunit region 173-204 inhibited α-bungarotoxin binding, and subsequent epitope mapping indicated that residues 183-194 are major determinants of toxin binding (Donnelly-Roberts and Lentz, 1989). The toxin binding sites are quite distinct from the main immunogenic region (MIR) targeted by an autoimmune response in the disease state of myasthenia gravis, which appears to be limited to the residues 67-76 on the α-subunit (Das and Lindstrom, 1989; Tzartos et al., 1988). These and other studies have helped to define specific binding regions on the AChR α-subunits.

Another less frequently utilized approach to determine the mechanisms of AChR-ligand binding is to use intact AChR and modify specific aspects of the ligand. This approach provides information about the functionally important portions of a ligand's structure. For example, Lapemis neurotoxin has three major structural domains defined by disulfides (loops A, B and C). When the sole tryptophan residue was chemically modified, the protein lost toxicity (Tu and Hong, 1971) and was unable to bind to the AChR (Allen and Tu, 1985). Tryptophan resides on loop B, indicating that this loop is essential to binding. Loss of toxicity may have resulted from the inability of the modified toxin to bind to receptor. By synthesizing each of the domain loops and assaying toxicity and binding, one should be able to define sequence regions necessary for receptor binding or for toxicity.

Low and Corfield (1987) presented a working theoretical model attempting to map the neurotoxin binding site on the acetylcholine receptor. The study pointed out that the differences in human receptor sequences and that of the Torpedo receptor may account for clinical differences in toxicity of sea snake and short chain α-neurotoxins with that of venoms containing long chain α-neurotoxins. However, this model did not consider or explain the other reported binding regions of the receptor known to participate in binding to α-neurotoxins.

The phosphorylation sites of the AChR subunits have been reported. Huganir and Miles (1989) discuss the sites of phosphorylation on the AChR subunits which are not at sites involved in acetylcholine or neurotoxin binding. They implied that an associated 43 kDa protein(s) are phosphorylating protein kinase(s), perhaps regulatory protein(s). LaRochelle et al.(1989) demonstrated that the 43 kDa protein(s) are absent from genetic variants of C2 muscle cells and that acetylcholine receptor expression is reduced.

The minimum number of lipids required for a functional AChR was determined and reported by Jones et al. (1988). The structures of the carbohydrate moieties linked to each AChR protein subunits were elegantly determined by Nomoto et al. (1986).

The chemical modification study by Mori and Tu (1988) demonstrated that the chemical linking of the free amino groups of the receptor subunits does not affect the binding of Lapemis toxin; however, Lin and Tu (1988) demonstrated that chemical linking of free sulfhydryl groups of the receptor subunits does reduce Lapemis toxin binding.

Lapemis toxin was first isolated from the venom of the sea snake Lapemis hardwickii by Tu and Hong (1971). Lapemis toxin was studied and the primary sequence structure determined by Fox et al. (1977). Chemical modification, toxicity and binding studies of the Lapemis toxin showed that the toxin belongs to the snake venom toxin class of Type I short chain postsynaptic α-neurotoxins. Type I α-neurotoxins consist of 60-62 amino acids and Type II being the long chain postsynaptic neurotoxins consisting of 71-74 amino acids (Tu, 1990; Lee, 1979).

Juillerat et al. (1982) published results of peptide synthesis of the Naja naja phillipines neurotoxin fragment (residues 16-48) that bound receptor although weaker than the intact neurotoxin.
They hypothesized the existence of a lethal "active center" of a cobra (Naja naja philippinensis) neurotoxin by synthesizing the matching residues 16-48 and showed the peptide to be highly active in binding to the AChR protein. However, the toxicity of the peptide was not indicated. The strength of binding of the

peptide was several-fold weaker than the intact neurotoxin yet slightly stronger than the normal ligand, acetylcholine. These results do suggest the existence of the active center but also suggest that other portions of the neurotoxin play either a direct binding role or structural role for the optimization of the neurotoxin's binding.

The major neurotoxin from Lapemis hardwickii venom (Lapemis toxin) inhibits binding of I-125 labeled α-bungarotoxin to the acetylcholine receptor by binding to the toxin sites on the α-subunit of the receptor (Allen and Tu, 1985). The three dimensional structure of Lapemis toxin is thought to be similar to other known three dimensional structures of sea snake neurotoxins (Allen and Tu, 1985) and will be useful for determining the mechanism of toxin binding to the AChR.

From this data, neurotoxin analogs may be designed to antagonize the neurotoxin and its effects and may serve as a nontoxic antigen to produce antibodies that recognize and neutralize the neurotoxin effects *in-vivo* thus serving as a toxoid to produce a vaccine. The designed antagonists may also, with appropriate studies, prove to be useful drugs in various aspects of neuromedical science studies and treatments. Envenomation by sea snakes is characterized by muscle paralysis and severe respiratory dysfunction, and occasionally results in death.

A synthetic approach to study the structure function relationship of Lapemis toxin was undertaken. By using solid phase peptide synthesis (SPPS) method the loop domains were made and their relative binding abilities to the AChR and their relative in-vivo toxicity determined.

The more that is known of this interaction, the more aspects of the basic research and medical research of the acetylcholine receptor and the neurotoxins can be studied.

The chemical structures of many snake postsynaptic neurotoxins have been identified, but the mechanism of binding to the nicotinic AChR is still not clear. In the past, most chemical modification was done on free toxins; however, the conclusions from such an approach are less reliable than methods using receptor-bound toxin. Comparing the results of chemical modification of AChR-bound neurotoxin with that of free neurotoxin will yield more precise information as to the role of a particular amino acid on toxin binding to AChR.

Recently Garcia-Borron et al. (1987) chemically modified lysine residues of the receptor-bound α-bungarotoxin instead of modifying unbound (free) neurotoxin. Reactivity of Lys-26, Lys-38, and other lysine residues to methylation is different when the toxin is bound to the receptor. It was concluded that Lys-26 binds to the nicotinic AChR.

By using the same approach, one can determine which of the three arginine residues in Lapemis toxin is most likely to bind to the nicotinic AChR. The modification of a number of arginine residues of the receptor-bound Lapemis toxin was investigated and compared with the modification of arginine residues of unbound (free) toxin. The positions of modified and unmodified arginine residues in the receptor-bound toxin and free toxins were identified from the amino acid sequence of fragments of these toxins.

Phospholipase A_2 is a hydrolytic enzyme common to most animal venoms (Rosenberg, 1990), and it is a component of sea snake venoms as well. Sea snake phospholipases have numerous potential biological activities, including neurotoxic, myotoxic and hemolytic activities. Considerable discussion has been generated concerning the relation of enzymatic and toxic activities of various phospholipases A_2 (see Rosenberg, 1990), and the role of enzyme activity to toxicity is not yet settled. Unlike other neurotoxic snake venom phospholipases, which possess pre-synaptic neurotoxicity, phospholipase A_2 from Laticauda semifasciata venom appears to exhibit post-synaptic neurotoxicity (Harvey et al., 1978; Harvey and Tamiya, 1980). The mechanism of this action has not been elucidated.

General tissue necrosis is not a typical manifestation of sea snake envenomation, but the occurrence of myonecrosis is apparent from the frequent observation of myoglobinuria in humans bitten by sea snakes (Reid, 1973, 1975a, b). Muscle damage resulted from the action of phospholipase A_2 in the venom of Laticauda semifasciata; however, this enzyme has low lethal toxicity (Tu and Passey, 1971). The enzyme from Enhydrina schistosa venom is both toxic

(Fohlman and Eaker, 1977) and myonecrotic (Sutherland et al., 1981), and it has several other biological activities as well (Tu, 1988). LD_{50} values for phospholipases isolated from sea snake venoms are given in Table 1-8.

Phospholipase A_2 enzymes in general promote the hydrolysis of diverse phospholipids at the SN-2 position. Substrate preferences/specificities vary depending on the source of the enzyme, but most common cell membrane phospholipids can serve as substrates. The specificity of their catalysis has made these enzymes useful probes of cell membrane and model membrane organization (e.g., Davidson and Dennis, 1991).

The catalytic action of phospholipases A_2 can lead to cell membrane damage and eventual cell lysis (Harris, 1985) via direct hydrolytic activity and via the autopharmacological action of the lysophospholipid products (Dennis et al., 1991). In addition, the release of fatty acids (such as arachidonic acid) may have a wide variety of secondary effects, such as the generation of lipid second messengers (Dennis et al., 1991). Differences in substrate specificity (and hence in products formed) may account in part for the large differences in toxicity among the single chain phospholipases A_2. The mechanism of action of the toxic phospholipase A_2 from Laticauda colubrina venom was not defined (Takasaki et al., 1988). However, a much less toxic phospholipase A_2 from L. semifasciata venom apparently acts in a fashion similar to the post-synaptic neurotoxins, binding to the acetylcholine receptor and preventing acetylcholine binding (Harvey and Tamiya, 1980). A myotoxic phospholipase A_2 from Enhydrina schistosa venom was shown to possess presynaptic neurotoxicity (Fohlman and Eaker, 1977), apparently the only known example of a presynaptic neurotoxin in sea snake venom.

Relatively few sea snake phospholipases A_2 have been sequenced, and like the α-neurotoxins, sea snake venoms typically contain several isoforms with one major toxin (Table 1-9). These enzymes consist of 118 amino acids and have a molecular weight of ~13,500 daltons. There are 14 half cystine residues, and the molecule is therefore very tightly compacted due to the highly conserved seven disulfides. These enzymes are remarkably heat-stable, and earlier work with venoms employed heating at >65C to inactivate other components (though neurotoxins were also not affected). One mole of calcium is bound per mole of enzyme, and activity is dependent on this cation (Teshima et al., 1989). The calcium binding site for homologous phospholipases A_2 has been shown to involve residues 28, 30, 32 and 49 (White et al., 1990); these are invariant residues in all snake venom phospholipases A_2, including an inactive homologue from Laticauda colubrina venom (Takasaki et al, 1988).

No sea snake phospholipases A_2 have as yet been subjected to x-ray diffraction studies. However, two homologous enzymes from other snake venoms have recently been crystallized and studied (Tomoo et al., 1989; White et al., 1990). The enzyme from Naja naja atra venom was crystallized in the presence of a transition state analog as a model of how the fatty acyl chains of the substrate are accomodated by the enzyme during catalysis (White et al., 1990). A tertiary structural model for the bovine phospholipase A_2 has been compared with a crotalid phospholipase A_2, and the backbone configurations are essentially identical (Renetseder et al., 1985). Global structural features of these studies can most likely be extended to sea snake phospholipases as well.

Phospholipase A_2 enzymes hydrolyze the fatty acyl chains of phospholipids at the SN-2 position. Amino acid side chains involved in hydrolysis of fatty acids include residues 48, 52 and 92 (White et al., 1990); these residues are also invariant for all active snake venom phospholipases A_2. An inactive phospholipase A_2 homologue from L. colubrina venom has asparagine 48 instead of histidine 48 (Takasaki et al., 1988); this substitution at the catalytic center is likely responsible for the lack of enzymatic activity. When histidine 48 of an active phospholipase A_2 from the same venom or from the venom of Notechis scutatus (Volwerk et al., 1974) is modified with p-bromophenacyl bromide, both enzymes lose activity and toxicity.

It has been demonstrated that phospholipases A_2 recognize polar head groups of several types of phospholipids rather than simply a hydrophobic environment, since Triton X-100 micelles lacking phospholipids will not bind phospholipase A_2 (Roberts et al., 1977). A "dual phospholipid" model has been proposed to explain

the activity of phospholipases A_2 on micelles and membrane vesicles (Hendrickson and Dennis, 1984a,b; Davidson and Dennis, 1991) which suggests that two phospholipid binding sites exist on the enzyme. One site is required for enzyme activation and the other includes the catalytic site. Phospholipase A_2 from L. semifasciata venom has also been shown to be activated by free fatty acids (Yoshida et al., 1979).

In the past, rates of hydrolysis of various phospholipids led to the ranking of substrate specificities for phospholipases A_2 from a given source (e.g., Adamich and Dennis, 1978). Since some phospholipases are extremely toxic while others are essentially non-toxic, and since phospholipid distribution in cell membranes is known to be asymmetric, it was hoped that substrate ranking would lead to the identification of particular "target" phospholipids. However, as has been noted (Rosenberg, 1990; Davidson and Dennis, 1991), seemingly small differences in assay procedures can give rise to strikingly different apparent substrate specificities, making it difficult to compare experimental results from different investigators.

A lively debate has continued for some years now as to the relation between phospholipase A_2 presynaptic neurotoxicity and enzymatic activity. Various investigators have shown that in some cases the pharmacological and catalytic activities could be dissociated (Karlsson, 1979; Yang et al., 1981; Rosenberg, 1986). However, phospholipases A_2 from different sources behave differently, and with some enzymes, catalytic and toxic activities appear to be linked. The exact mode of action of toxic phospholipases A_2 at the neuromuscular junction is unknown. However, at least one phospholipase A_2 (from L. semifasciata venom) appears to act like an α-neurotoxin by binding to the acetylcholine receptor and preventing acetylcholine uptake (Harvey and Tamiya, 1980). Other sea snake venom phospholipases A_2 do not appear to interact with the acetylcholine receptor.

Neurotoxins from sea snake venoms have been extremely useful tools for elucidating the structure and function of the mammalian acetylcholine receptor. These toxins are a dominant component of the venoms and are reasonably simple to isolate. Previous reports from this lab have detailed the elucidation of primary structure and secondary structure of several of these toxins, particularly Laticauda toxin. These short-chain α-neurotoxins are quite similar in amino acid sequence and pharmacological action, and the sequence of over 30 such proteins is now known. Recently, a signal peptide sequence was determined from cDNA encoding several toxins from the venom of Aipysurus laevis. It appears likely that most α-neurotoxins are first translated as precursors and are secondarily modified. However, nothing is known of the mechanism of processing of the precursor, and a free protoxin form has not been demonstrated.

Water-soluble extracts from Laticauda hardwickii venom glands have been used as a source of neurotoxin and phospholipase A_2 and likely contains precursor toxin if it is present in detectable quantities in the gland. We have previously isolated a 9 kilodalton protein which coelutes from gel filtration columns with the phospholipase A_2. The signal peptide of Aipysurus toxins are each 21 amino acid residues in length, and Laticauda toxin consists of 60 amino acid residues. An 81 amino acid protein would have an apparent molecular weight very close to 9 kD, and the properties of this protein were investigated further.

Gila toxin was first isolated and basic characterization was reported by Hendon and Tu (1981) Biochemistry 20: 3517-3522. However, the primary structure was not determined. The toxic principles of gila monster have not been clarified yet. Gila toxin, a main toxic principle of gila monster venom was isolated but its nature of toxic aciton and chemical properties have not been studied yet.

The primary goal of this study is to determine the primary sequence of gila toxin (Heloderma horridum) and examine whether the toxin is a prostsynaptic neurotoxin or not. This information is of critical importance to evaluate the structure/function relations of specific toxin domains. Gila toxin has a relative molecular weight of 37,500 and does not possess phospholipase activity, proteolytic activity, nor hemorrhagic activity; however, gila toxin has significant lethal activity, with an LD_{50} of 2.75 μg/g. The toxin's mechanism and sites of action are still unknown. From the results of this work the primary sequences can then be used to compare to the other known toxin sequence in an effort to pinpoint the mode of action. Results will also suggest future

experiments to study this unique reptilian toxin.

Among all types of lizards, the Gila monsters are the only genus possessing venom. There are 2 species of Heloderma: H. suspectum and H. horridum. H. suspectum comprises 2 subspecies: H. suspectum cintum and H. suspectum suspectum. Heloderma horridum is generally acknowedged to comprise 3 subspecies: H. horridum exasperatum, H. horridum horridum and H. horridum alvarazi.

Gila monsters (Heloderma) are found only in North America, ranging from southern Mexico to Utah and the southern tip of Nevada in the United States (Tu, 1991). Helodermatids are large and relatively slow-moving. Most adults measure between 300 and 400 mm. Gila monster venom glands are inferior labial glands located on each side of the lower jaw, in contrast to the venom glands of venomous snakes, which are situated in the upper jaw (Bogert, 1981). The introduction of venom does not utilize hollow or hypodermic-like fangs. The venom is transferred from the venom glands to the venom ducts, which then discharge their contents on the mucous surface near the base of the teeth. The venom is drawn into the puncture wound, made by the teeth, through the capillary action along the grooved surface of the teeth.

Venom from Gila monster contains many different kinds of proteins and enzymes which are toxic to many animals. For example, toxic phospholipase A_2 in the venoms of H. suspectum and H. horridum were found to have an molecular weight of 19,000 with 170 amino acid residues (Sosa and Alagon, 1986). Comparision of partial amino acid sequence of Gila monster venom phospholipase A_2 with bee venom and snake venom enzymes also indicated some sequence homology.

Hyaluronidase or spreading factor had been isolated from H. horridum (Tu and Hendon, 1983). It was shown that hyaluronidase promotes the distribution of the hemorrhagic area in mice that have been injected with a hemorrhagic toxin, indicative of this action as the "spreading factor"(Tu and Hendon, 1983). Gila monster venom also contains many different kinds of proteolytic enzymes, some of which have been purified and characterized. For example, the 65,000 dalton protein is responsible for hydrolysis of N-benzoyl-L arginine ethyl ester (BAEE) (Alagon and Maldonado, 1982). Like many snake venoms, Gila monster venom also causes hemorrhage upon envenomation. A hemorrhagic toxin was isolated recently and characterized (Nikai et al., 1988). This protein has molecular weight of 31,000 and pI of 3.9. This toxin shows arginine ester-hydrolyzing activity with lethal action (LD_{20}=0.38 $\mu g/g$).

All of the proteins that have been described so far are also found in the snake venoms. However, Gila monster venom also contains unique proteins, some of which have been isolated and charactereized. For example, Helodermin (vasoactive intestinal peptide like peptide). This peptide has molecular weight of 5,900 and has an amino acid sequence homologous to VIP (vasoactive intestinal peptide). Helodermin is shown to bind to VIP receptor leading to activation of adenylate cyclase and an increase in cellular cAMP level in rat pancreatic cells (Vandermers, and Gourlet, 1987).

Gila toxin Gila toxin is a unique protein that has been isolated from the venoms of H. suspectum and H. horridum (Tu and Hendon, 1981). The toxin from both species show similar amino acid composition, electrophoretic mobility, pI, and immunological reactivity. They are glycoproteins, having relative molecular weights of 35,000 - 37,500. These proteins are single polypeptide chains and have isoelectic points of 4.2. Neither is antigenically related to the venom of snakes. Physically and chemically, Gila toxin most closly resembles hemorrhagic toxin C from Crotalus atrox and crotoxin from Crotalus durissus terrificus (Tu and Hendon, 1981). Common characteristic includes molecular weight in excess of 20,000 and acidic isoelectric point. However, Gila toxin is devoid of phospholipase A_2 activity and posses no known proteolytic, hemorrhagic nor hemolytic activity. Also, the LD_{50} of Gila toxin (2.5 $\mu g/g$) is considerably higher relative to the presynaptic neurotoxin crotoxin and postsynaptic neurotoxin (cobra toxin); both have LD_{50} values of 0.06 $\mu g/g$ and gross observation of envenomated animals shows neurotoxic symtoms. However, when combining Gila toxin with other venom components, the LD_{50} was lower than that of individual fractions. This was highly suggestive of a synergistic action with other venom components.

Section 2.

Materials and Experimental Methods

Materials. Sephadex G-50 and G-10, Cm-cellulose, CNBr-activated Sepharose 4b, and molecular weight standards were purchased from Sigma Chemical Co. Torpedo californica electroplax, excised and then quick frozen in liquid nitrogen, was obtained from Pacific Bio-Marine Laboratories (Venice, CA) and stored at -70°C until needed. ^{125}I-α-bungarotoxin and Econofluor were purchased from New England Nuclear (Boston, MA). Phenylglyoxal monohydrate was purchased from Aldrich Chemical Co. (Milwaukee, WI). Phenyl [2-^{14}C]glyoxal was obtained from Research Products International Corp. (Mount Prospect, IL). Endoproteinase Glu-C (protease V8) was from Boehringer Manheim Biochemicals (Indianapolis, IN). Extracti-Gel D and Phenylisothiocyanate (PITC) were purchased from Pierce Chemical Co. (Rockford, IL). All sequence and peptide synthesis reagents were purchased from MilliGen/Biosearch (Burlington, MA). all other chemicals were of analytical or reagent grade.

Fmoc-peptide synthesis. The 9-fluorenylmethoxycarbamoy (Fmoc) protected peptides were used to synthesize the desired peptides using the MilliGen 9050 peptide synthesizer and manufacturer protocols. The method is discussed in detail by Paivianan et al. (1987). The main peptide reagents and protected amino acids were purchased from MilliGen. The progress of the deprotection and acylation (chain elongation) steps were monitored at 365 nm. Shown in figure 2-1 is an example of the chemistry involved in one cycle of peptide synthesis. Step 1 is the deprotection of the first amino acid that is already covalently attached to the solid support resin. PIP 20% in DMF will remove the Fmoc group yet not deprotect the side chain of the residue. In this example the cysteine residue side chain is protected by a trityl group. Note that the synthesis proceeds from the carboxyl terminal of the intended peptide toward the amino terminal. Once the Fmoc group has been removed the exposed amino group is ready for the coupling reaction Step 2. The next activated amino acid with either the OPFP ester or ODhbt ester is added with the HOBt in DMF and allowed to react and couple. In this example the activated amino acid was glycine. Following step 2, the synthesis repeats step 1 and 2 until the entire peptide has been completed. Once completed the entire peptide is then cleaved from the support resin and side chains deprotected in one step and the peptide separated from the reaction mixture and resin.

Cleavage of Fmoc Peptides from Polyamide Resins. Fmoc synthetic peptides were cleaved from the solid phase resin using 90% trifluoroacetic acid (TFA), 5% thioanisole, 3% ethanedithiol, and 2% anisole for 8 to 10 hours. The cleavage solution containing the released peptide was removed from the resin by vacuum filtration through a glass fritted buchner funnel. The cleavage reagent was remove using a rotoevaporator at 55° C for 20 to 30 minutes leaving a brown thick syrup residue containing the peptide. The next step was to precipitate the peptide with 3X wash of anhydrous diethylether and the ether layers removed. The peptides were then allowed to dry by evaporation of the residue ether. The solid peptides were then resuspended in HPLC grade water with a few drops of acetic acid to aid solubility. This solution was pipetted to a tared vial, froze, lyophilized and weighed. The peptides were then subjected to further analysis and purification by RP-HPLC.

Purification of Peptides. Each of the synthetic peptides was purified by injecting aliquots of the peptides in a Beckman ultrasphere-ODS column (4.6 mm x 25 cm with 5μ particle size and 80 Å pore size) containing a guard column of ultrasphere-ODS (4.6 mm x 4.5 cm with 5μ particle size and 80 Å pore size) operated at a flow rate of 0.8 mL/min. Gradient conditions: 90% solvent A (0.1% TFA) and 10% solvent B (0.1% TFA in acetonitrile) for 5 min. Then, a linear gradient from 10% solvent B to 90% solvent B in 30 min. Then, holding at 90% solvent B for 10 min. followed by return to 10% solvent B in 0.1 min and holding for 10 min. Absorbance at 214 nm was monitored. Each of the peptides were diluted to approximately 1 μg/ml and allowed to sit for 24 h at room temperature. This was done to favor intramolecular disulfide formation over intermolecular formation.

Amino Acid Sequencing of Peptides. Each of the five synthetic peptides was subjected to automated Edman degradation and sequencing using the Milligen Model 6600 ProSequencer and manufacturer protocols. The Arylamine membrane discs provided by Milligen were used to covalently attach the peptides before sequencing. Each peptide was dissolved in 30 % acetonitrile (solution A) and a total of 20 µl was spotted in 10 µl aliquots on the Sequelon-AA membrane disc and placed on a sheet of mylar that rested on a heat block set at 55 °C and allowed to dry. The membrane was removed and allowed to cool. Next, 5 µl of solution B, containing 10 mg/ml of N-(dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) in 0.1 M 4 morpholineethanesulfonic acid (MES), pH 5.0 and 15 % acetonitrile, was applied to the membrane containing the dried peptide and allowed to react for 20 minutes at room temperature. The membrane disk was then placed in the Prosequencer for automated Edman chemistry sequencing of the peptides. The Edman reagent (PITC) is added at pH 8-9 and allowed to react. The amino terminal residue cleaves from the protein or peptide and reacts with the PITC to form the PTC derivative of the amino terminal residue. Under acidic conditions the ATZ derivative is formed as an intermediate to forming the final PTH derivative. The shortened sample is recycled for the next Edman reaction. The PTH derivative is chromatographed (HPLC) and identified by comparison to PTH standards.

Reduction and Alkylation of Peptide B1. Peptide B1 (1 mg) was weighed and dissolved in 1 ml of 0.1 M Tris buffer (pH 8.6). This solution was divided into two equal 500 µl portions with one aliquot subjected to HPLC and used as a control. From this same aliquot 50 µl was subjected to Ellman's reagent (DTNB) to assess the status of the terminal cysteine residues. For a control in this assessment 100 mM DTT solution was serially diluted to serve as a calibration of the sensitivity of the Ellman's reagent in detecting free sulfhydrl groups. The other 500 µl aliquot was mixed with 500 µg of DTT, flushed with nitrogen gas, capped tightly and incubated at 50° C for 2 h. A control buffer blank of 500 µl was similarly treated. After incubation, 50 µl of 0.2 M Iodoacetamide in 0.1 M Tris buffer (pH 8.6). After two minutes 100 ul was added drop wise. After another two minutes again another 100 ul was added. To assess the disappearence of the SH groups 50 µl of reaction mixture was mixed with 50 µl of Ellman's reagent (0.02 M 5,5' dithio bis(2-nitro) benzoic acid in 95% ethanol). The unmodified peptide B1 aliquot, the reduction alkylation mixture and the control buffer blank were subjected to analytical RP-HPLC analysis. The carboxyamidomethylated (CA) peptide B1 was futher purified for binding studies using RP-HPLC. Figure 2-2 illustrates the carboxyamidomethylation of peptide B1.

Venoms. *Naja naja atra* (Taiwan cobra) venom was collected in Taiwan and stored lyophilized at -20 °C until needed. Sea snakes, *Lapemis hardwickii*, were captured in the Gulf of Thailand in 1986 and 1989, and the venom was extracted as previously described (Tu & Hong, 1971). Glands were dissected out and dried in a cool room. Dried glands were then pulverized to a coarse powder using a Wiley mill. A total of 470 g powdered glands was obtained for venom extraction. Cold glass-distilled water was added to approximately 20 g dried gland powder, and the resulting paste was ground (using a mortar and pestle) for 40 min at 4 °C. The paste was then suspended in approximately 600 ml cold distilled water, stirred vigorously and allowed to sit for 10 min at 0 °C. The suspension was then stirred, divided into two 400 ml centrifuge bottles and was centrifuged for 20 min at 4000 rpm (4 °C). The supernatant was collected, shell-frozen and lyophilized, and the remaining solids were ground and extracted again (as above). Approximately 46.5 g of gland extract (crude venom) were obtained from 470 g dried glands or an approximate yield of 10% by weight.

Lapemis toxin (Neurotoxin). All isolations followed the previously published methods of Tu, et al. (1975) based on Tu & Hong (1971). The major neurotoxin was isolated using a two-step gel chromatography procedure at 4 °C. First, venom was loaded onto a Sephadex G50-50 column (2.5 x 100 cm) that had been previously equilibrated with a 0.01 M sodium phosphate buffer (pH 6.5) containing 0.1 M NaCl. The toxin was eluted with the same buffer at a flow rate of 14 mL/h, and the eluate was collected in 3 mL aliquots.

The tubes that included protein peak IV were pooled and lyophilized. The samples were dialyzed (MWCO 1000 SpecPor) against cold glass distilled water (3X

changes) over 24 h or desalted on Sephadex G-10 or Biorad P-10 columns.

The lyophilized toxic peak fraction was applied to a CM-cellulose column (1.5 x 45 cm) previously equilibrated with 0.01 M sodium phosphate buffer (pH 7.8). The toxin was eluted with a linear gradient of NaCl from 0.0 to 0.4 M in the phosphate buffer. The tubes containing the toxic protein were pooled, dialyzed, and lyophilized.

Toxicity was checked after each step of the isolation procedure. After G-50 chromatography, three to five Swiss Webster mice were injected with protein from peak III dissolved in 0.9% NaCl at a concentration that was twice the LD_{50} of crude venom. The number of mice that died in 24 h was recorded.

The homogeneity of the toxin was checked using polyacrylamide gel electrophoresis (SDS-PAGE) with the Pharmaceia PhastSystem 8-25% gradient gels under reducing conditions and stained with Coomasie blue or silver stained.

Isolation of Cobrotoxin. The venom from *Naja naja atra* was subjected to Sephadex G50-50 gel filtration pre-equilibrated with 10 mM sodium phosphate buffer (pH 6.5) containing 0.1 M NaCl. Peak IV containing the neurotoxin was pooled, dialyzed and lyophilized. This lyophilized fraction was then subjected to CM-cellulose or CM-sephadex C25 ion exchange chromatography pre-equilibrated with 10 mM sodium phosphate buffer (pH 7.8). After one column volume of buffer had been eluted a linear salt gradient from 0 M NaCl to 1 M NaCl (in 800 ml) was applied. The first major peak after the gradient was applied was determined to be the neurotoxin after gel electrophoresis, amino acid analysis and toxicity checks. This method is essentially that of Tu et al. (1975) based on Yang (1965, 1969).

The amino acid analysis of the cobrotoxin was done using the PTC amino acid derivatization method. The sample was hydrolyzed using 6 N constant-boiling HCl at 110 °C under nitrogen in sealed ampules for 24 h. The sample was dried under vacuum, resuspended in 20 μl of neutraliztion buffer (0.7 ml of absolute ethanol, 0.1 ml of triethylamine and 0.1 ml of Nanopure water), vortexed and redried under vacuum. Coupling buffer (0.7 ml of absolute ethanol, 0.1 ml of triethylamine, 0.1 ml of Nanopure water and 0.1 ml of PITC) 20 μl was added, and the mixture was allowed to react at room temperature for 20 min and evaporated to dryness. The resulting phenylthiocarbamyl amino acids were dried and analyzed on a Waters chromatography Picotag HPLC (Waters Chromatography Division).

AChR Isolation. AChR was isolated from *Torpedo californica* electroplax tissue (rich in AChR) using the method of Froehner and Rafto (1979) and later the method of Lindstrom et al., (1980). Cobrotoxin affinity resin was prepared as previously described (Brockes and Hall, 1975) using CNBr-activated Sepharose 4B. Re-isolation of the *Torpedo californica* nicotinic acetylcholine receptor was accomplished using an alternate procedure reported to give a more stable and active receptor. The method was used with only slight modification. The details follow:

All procedures were carried out at 4°C. *Torpedo californica* electroplax tissue (rich source of receptor) (75 to 88 g) was cut into pieces using a #11 scalpel. The tissue pieces were mixed with 200 ml of buffer A. Buffer A consisted of 10 mM sodium phosphate, pH 7.5, containing 10 mM sodium azide (inhibitor of bacterial growth), 5 mM EDTA (inhibitor of metalloproteases), 5 mM iodoacetamide (inhibitor of cysteine proteases) and 1 mM phenylmethylsulfonyl fluoride (PMSF) (inhibitor of serine proteases). The mixture was then blended on high speed in a waring blender (8 times for 15 sec each). The mixture was strained through a wire mesh. The mixture was centrifuged for 10 min at 5000 rpm in Beckman Ti60 rotor (4°C) (1,775 x g). The supernatant was centrifuged for 60 min at 19,000 rpm (25,634 X g) using the same rotor. The pellet of this spin was then resuspended with a Dounce homogenizer in buffer B. Buffer B consisted of 10 mM sodium phosphate, pH 7.5, containing 10 mM sodium azide (NaN_3), 1 mM EDTA, soybean phospholipid 5mg/ml (stabilizing lipid), and sodium cholate 2% (w/v) (detergent to solubilize the receptor). The resuspension was mixed with a Virtis mixer for 30 min. The mixture was then centrifuged for 30 min at 30,000 rpm (63,907 x g) in the Beckman Ti60 rotor. Affinity chromatography of the supernatant was the next step. Cobrotoxin, isolated previously from the cobra venom, was treated with the CNBr-activated sepharose 4B resin to give the affinity resin. The supernatant was mixed gently by the Virtis mixer with the

toxin-linked resin for 1 hour. The columns were then poured. The columns were washed with 250 to 300 column volumes of buffer C. Buffer C consisted of 4 mM sodium phosphate, pH 7.5, containing 100 mM NaCl, 5mg/ml soybean phospholipid, and 2% (w/v) sodium cholate. The resin was then transferred to a beaker containing 25 ml of receptor elution buffer D and mixed for 12 to 15 h with the Virtis mixer. Buffer D consisted of buffer C containing 1 M carbamoylcholine chloride. The resin was repoured into the columns and the eluant containing the purified acetylcholine receptor was collected. The receptor eluant was then dialyzed (dialysis tubing MWCO 6,000 - 8,000) against 500 volumes of buffer E for 24 h. Buffer E consisted of 10 mM sodium phosphate, pH 7.5, containing 100 mM NaCl, and 0.1 %(w/v) sodium cholate. The receptor eluant was then dialyzed against 500 volumes of buffer F for 24 h. Buffer F consisted of 10 mM sodium phosphate, pH 7.5, containing 0.02% (w/v) lauryl sulfate (SDS), and 0.04% (w/v) sodium cholate. The dialysate was then aliquoted into Eppendorf tubes and frozen at -70 °C until needed. An aliquot was subjected to the Pierce BCA protein concentration determination assay. Another aliquot was subjected to SDS-PAGE and stained with Coomassie blue for a purity check. The purified receptors were then used in the binding studies. Figure 2-3 shows the above protocol in a flow diagram form. RCF (Relative Centrifugal Field) = $rw^2/g = 1.12\ r\ (rpm/1000)^2$; g=9807 mm/s^2; w=angular velocity in radians per second ($2\pi rpm/60$); r is radius in millimeters; r_{av}=63.4 mm for Beckman Ti60 rotor.

Radioiodination. [125-I] Radiolabelling of α-Bungarotoxin (α-Bgtx), Lapemis Toxin (LTX) and Tyrosine Containing Synthetic Peptides. The α-bungarotoxin (10 μg), lapemis toxin (10 μg) and each peptide (5 μg to 10 μg) were labelled using the Chloramine-T method with 1 mCi of $Na^{125}I$ under optimized conditions to favor iodination of the tyrosine residue only. The procedure uses a 0.01 M sodium phosphate buffer (pH 7.5) to dissolve each peptide. To this solution 1 mCi of NaI^{125} was added. Chloramine-T was also added and the reaction was allowed to take place for 2 min at room temperature. The reaction was stopped using sodium betametasulfite. The reaction mixture was then applied to a Sephadex G-25 desalting column to separate the labelled peptide from the reagents. The amount of activity incorporated was measured. These solutions were then used in the binding studies.

Competion Binding Assay. Binding of the synthetic peptides to the nicotinic acetylcholine receptor was determined by their ability to compete with Lapemis ^{125}I-neurotoxin. Toxin binding to receptor was determined using the method of Schmidt and Raftery (1973). Assays were done in 10 mM sodium phosphate, pH 7.0 containing 100 mM NaCl and 0.1 % Triton X-100 or in *Torpedo* Ringer's buffer containing 250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 5 mM sodium phosphate (pH 7.0), and 0.1% bovine serum albumin at room temperature in a total volume of 125 μL, for 1 h. Two Whatman DE-81 filter disks were placed on a Millipore filter holder connected to a vacuum flask and were rinsed with a wash buffer containing 100 mM NaCl, 0.1% Triton X-100, and 10 mM sodium phosphate (pH 7.4). Then 50 μL of the incubation mixture was applied to the DE-81 filter disks and allowed to soak for 10 min. The disks were then rinsed five times with 5 mL of buffer. The filters were placed in 5 mL of Econofluor, and counted in a Beckman LS 7800 liquid scintillation counter. The binding of the synthetic peptides were checked by determining the decrease in ^{125}I-neurotoxin binding to the receptor following preincubation of the receptor with varying amounts of synthetic peptide for 1 h. In other words, ^{125}I-neurotoxin was used as a nonreversible back-titrant to measure specific binding of a ligand competing for the same receptor (Juillerat et al., 1982).

Direct Binding Assay. The [125-I] labelled peptides binding studies employed the DE-81 filter paper assay of Schmidt and Raftery (1973) or as modified by Allen and Tu (1985). Assays were done in 10 mM sodium phosphate, pH 7.0, containing 100 mM NaCl and 0.1% Triton X-100 or Torpedo Ringer's buffer containing 250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM sodium phosphate (pH 7.0), and 0.1% (w/v) bovine serum albumin at room temperature. The amount of receptor was selected to give a final concentration of 2 nM in the incubation mixture. Labelled peptides were titrated into the incubation mixture with the final volume of each assay being 250 μl. The solutions were allow to incubate for 1 h. Then, 100 μl of the incubation mixture was applied to the Whatman DE-81

filter paper disc previously placed in a Millipore vacuum filter apparatus and allowed to soak in for 10 min. The disc was then rinsed five times with 5 ml of wash buffer. The wash buffer consisted of 10 mM sodium phosphate, pH 7.4, containing 100 mM NaCl and 0.1% (v/v) Triton X-100. Each filter disc was then place in a scintillation vial containing 5 ml of Econofluor and counted in a Beckman LS 7800 liquid scintillation counter. Background counts due to non-specific binding were determined by doing the same assay with no receptor added.

Screening Toxicity Assay of the Synthetic Peptides. Two 25 gm female Swiss Webster mice for each crude peptide sample was injected intravenously with 0.1 cc of a 2 mg/ml solution of each sample. The mice were observed for twenty four hours for adverse clinical signs and lethality. The toxicity check of peptide B1 was repeated using three mice at the same dose and observed for three days for any ill affects. After three days the animals were euthanized with nitrogen gas and subjected to necropsy examination. Two normal control mice were likewise treated for necropsy comparison.

Hydrophilicity analysis. Since the amino acid sequence is known for Lapemis toxin the hydrophilicity analysis was possible. A computer program based on Hopp and Woods (1983) was used to evaluate the hydrophilic regions of the toxin. Essentially, the program assigns to each amino acid a hydrophilic value ranging from a high value of 3.0 for hydrophilic amino acids to -3.4 for the hydrophobic residues. These values are based on solvent parameters determined by Levitt (1976). Table 2-1 lists and compares the Hopp Woods hydrophilicity values (H-values) to the Levitt amino acid solvent parameters. The single letter sequence is entered and the program assigns the average H-values based on a six amino acid moving window through the length of the protein. This length was determined to be the best for predicting antigenic response to viral coat proteins with high correlation between high hydrophilic value regions and high antigenicity response.

Amino Acid Analysis. The analyses were performed on a Beckman Model 344 M HPLC system using reverse-phase column after precolumn derivatization by PITC. The preparation of the PTC amino acids was as described by Heinrikson and Meredith (1984). Derivatives were resolved and applied on a Beckman ultraphsere-ODS column (4.6 mm x 25 cm) operated at a flow rate of 0.9 mL/min. PTC derivatives were detected by the absorbance at 254 nm. Samples were hydrolyzed for 24 h and 48 h at 110-115°C in 6 N HCl, sequanal grade.

SDS-PAGE. Electrophoresis was performed on SDS-polyacrylamide gel (10%) using a procedure modified from Laemmli (1970). The sample buffer contained 2%SDS and 5% β-mercaptoethanol. Samples were boiled for 5 min before electrophoresis. Bovine serum albumin (66,000), egg albumen (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (20,000), and α-lactalbumin (14,200) were used as standards for molecular weight determinations (Weber and Osborn, 1969) Receptor and standards were mixed and applied to the same well for molecular weight determination. Gels were stained with Coomassie brilliant blue and destained.

Arginine Modification. Modification of arginine residues in Lapemis toxin with phenylglyoxal was performed by previously published procedures (Takahashi, 1968; Yang et al., 1974) with minor modification. The commercial ^{14}C-labeled phenylglyoxal employed throughout this study had specific activities of 25-35 mCi/mmol and was freed of impurities by chromatography on Silicagel 60.

To a solution of Lapemis toxin (0.6 μmol) in 1 mL of 0.2 M N-ethylmorpholine acetate buffer (pH 8.0), a 100-fold molar excess of phenylglyoxal in 0.3 mL of the same buffer was added, and then the reaction was allowed to proceed at 27°C for 3 h. The mixture was passed through a column of Sephadex G-10 (2.5 x 50 cm) followed by CM-cellulose chromatography with a linear gradient of increasing salt concentration from 0.0 to 0.4 M NaCl in 0.01 M ammonium acetate, pH 6.8. The fractions of the main protein peak were lyophilized and desalted through a column of Sephadex G-10 (2.5 x 50 cm) equilibrated with 1% acetic acid. The protein fractions were then pooled and lyophilized.

Identification of Arginine Residues Modified by Phenylglyoxal.

In order to determine the position of the modified arginine residues, the modified derivatives were digested with endoproteinase Glu-C (protease V8) after reduction and S-carboxymethylation. Reduction and S-carboymethylation were

performed by the method described by Crestfield et al. (1963). The S-carboxymethylated derivatives were dissolved in 0.02 M $NaHCO_3$ buffer (pH 7.2) to give a 1% solution, and endoproteinase Glu-C (50:1) was added. Digestion was carried out at 27°C for 16 h.

Arginine-containing peptides from endoproteinase Glu-C digests were separated by RP-HPLC (Beckman ultrasphere-ODS, 4.6 mm x 25 cm). Gradient conditions were as follows: from 95% solvent A (0.1%TFA) and 5% solvent B (0.1% TFA in acetonitrile) to 40% solvent B in 40 min. Peptides were detected by their absorbance at 214 nm. The eluting peptides were collected manually, dried, and stored at -20°C until needed for amino acid analysis.

Arginine Modification of Lapemis Toxin Part in the AChR Complex. The AChR (5 mg) from cobrotoxin-affinity chromatography was concentrated by ultrafiltration using Centriflo membrane cone (MW cutoff 25,000 daltons, Amicon Corp.). Ultrafiltration was carried out with 750 g at 4°C, and reduced 10 mL initial volume to 2 mL. In an arginine-modification experiment, the required amount of AChR was mixed with a twofold excess of Lapemis toxin. After 1 h incubation at room temperature, AChR solution was applied onto a Sephadex G-50-80 column (2.5 x 90 cm) previously equilibrated with 0.2 M N-ethylmorpholine acetate buffer (pH 8.0) containing 0.1% Triton X-100. The AChR-bound Lapemis toxin peaks were pooled and concentrated by ultrafiltration using Centriflo membrane cone. Chemical modification of bound neurotoxin was carried out by using [^{14}C]phenylglyoxal was added, and then the reaction was allowed to proceed at 27°C for 3 h. The mixture was dialyzed using Spectrapor membrane tubing, MW cutoff 12,000- 14,000, for 12 h against 0.01 M ammonium acetate, pH 6.8.

The labeled Lapemis toxin was released from AChR by adding 6 M NaCl and incubating at 37°C for 6 h. Separations of Lapemis toxin from AChR and removal of 0.1% Triton X-100 from the reaction mixture were carried out by using an Extracti-Gel D, detergent-removing gel, column (0.8 x 1.5 cm). The homogeneity of the toxin was checked by SDS-PAGE with Phast Gel Gradient 8-25% using Pharmacia PhastSystem, and also by RP-HPLC. The concentration of the Lapemis toxin was determined by using a MicroBCA protein assay kit (Pierce Chemical Co.) with bovine serum albumin as a standard.

Isolation and Search for Lapemis Toxin Precursor.

Fractionation. Venom was applied to a 94 cm X 2.5 cm Sephadex G-50-50 column (500 mg in 4.0 ml buffer) as described previously (Tu and Hong, 1971). Peak II (fractions 88-105) were pooled, dialyzed against distilled water (2 X 1000 ml) and lyophilized. This material was dissolved in 5.0 ml 10 mM tris/HCl pH 8.0 and applied to a 35 cm X 1.5 cm column of DEAE-Sephacel previously equilibrated with the same buffer. A gradient of 0-0.4 M NaCl in buffer was started after fraction 30. The main peak (IV) fractions were pooled, dialyzed against distilled water and lyophilized. This material was redissolved in 2.0 ml of 50 mM HEPES buffer pH 7.5 containing 200 mM NaCl and was applied to a 90 cm X 1.5 cm P-10 column. Fractions 30-45 were combined, dialyzed and lyophilized. Purity of this preparation was assessed by SDS-PAGE. Phospholipase A_2 (PLA2) was purified from Lapemis hardwickii venom from Peak II of the initial gel filtration step (Sephadex G-50) which was subjected to ion exchange chromatography (DEAE-Sephacel) followed by a second gel filtration step (BioGel P-6 or P-10). PLA2 was homogeneous as judged by SDS-PAGE.

Molecular Weight and Isoelectric Point Estimates. Relative molecular weight estimates of various fractions were obtained from SDS-PAGE using a Phast electrophoretic system and molecular weight standards (Pharmacia). All gels utilized a Laemmli buffer system (Laemmli, 1970) and samples were reduced with β-mercaptoethanol. Protein samples dissolved in distilled water were subjected to isoelectric focussing on the Phast system and pI was estimated from comparison with standards.

Determination of Phospholipase A2 Activity. Activity of the protein obtained from the BioGel P-10 column was assayed by the method of Wells and Hanahan (1969). Dipalmitoyl phosphatidylcholine was dissolved in ether:methanol (95:5) at 15 μmol/ml. Protein samples (25 μg in 50 μl of 0.2 M NaCl, 20 mM CaCl2) were added to 2.0 ml substrate solution, vortexed for 30 sec and incubated at RT (~22 C) for 15 min with rapid stirring. The

reaction was quenched with 3.0 ml 95% ethanol and 20 µl 0.1% cresol red dye was added. Released fatty acids were titrated with 20 mM NaOH in 90% ethanol.

Determination of Phospholipase A2 Activity

Reduction and alkylation of cystines. One milligram of PLA2 (~74 nmol) was dissolved in 1 ml 100 mM tris/HCl pH 7.5 containing 1% SDS. The solution was overlain with N_2 and boiled for 2 min. A 50 M excess of crystal dithiothreitol (based on 16 cysteine residues) was added, vortexed, overlain with N_2 and boiled for 3 min. The solution was again overlain with N_2 and reduction was allowed to proceed for 1.5 hr at room temperature (~22 C). A 200 M excess of 4-vinylpyridine was then added to the solution and alkylation proceeded overnight at room temperature. The solution was then dialyzed against 2 liters of 10 mM ammonium bicarbonate pH 7.5 containing 0.01% SDS for 24 hr followed by dialysis against 0.001% SDS for 24 hr. After lyophilization, excess SDS was removed by ion pairing. SDS was extracted by shaking with 1.0 ml acetone, triethylamine, acetic acid and water (85:5:5:1, v/v).

Cyanogen bromide fragmentation. PLA2 was dissolved in 0.5 ml 70% trifluoroacetic acid at 2.0 mg/ml. Cyanogen bromide (100 M excess) in 0.5 ml 70% TFA was added and the reaction was incubated at 37 C in the dark for 24 hr. The reaction was then quenched with 10 volumes of water, frozen and lyophilized. The lyophilizate was redissolved in 5 ml water, frozen and relyophilized (2X). Cleavage at methionine residues was evaluated by SDS-PAGE. Fragments of 10 kD, 8 kD and ~2 kD were observed, with the 8 kD band being the dominant cleavage fragment.

Electroelution of 8 kD band. After repeated lyophilization, the CNBr cleavage products were subjected to SDS-PAGE using a tricine buffer system and a Novex Corp. electrophoresis apparatus. Electrophoretic separation took ~2 hr; the gel was then briefly stained with Coomassie blue R-250, destained and the bands of interest were excised with a razor. The band corresponding to the 8 kD fragment was removed from 6 gel lanes and placed into an electroeluter (Hunkapillar design) fitted with 3.5 kD dialysis membranes. Electroelution was performed overnight (~15 hrs) at 1-2 mA. Electrophoretic dialysis against 10 mM ammonium bicarbonate buffer (pH 8.5) was performed in the electroeluter. The eluted sample (~100 µl) was lyophilized and Coomassie and SDS were extracted by shaking with 1.0 ml acetone, triethylamine, acetic acid and water (85:5:5:1, v/v). Final yield of the 8 kD band was ~ 20 µg.

Fragmentation of 8 kD band with Arg-C endoproteinase. Approximately 10 µg of the 8 kD fragment was dissolved in 50 µl of 50 mM tris pH 8.0 containing 8 M urea, 3 mM dithiothreitol and 50 mM NaCl and boiled for 3 min. After cooling, 150 µl of 50 mM tris pH 7.6 (containing 2 mM Dtt, 1 mM $CaCl_2$ and 50 mM NaCl) was added. One microgram of Arg-C endoproteinase (Boehringer-Mannheim) was added and the solution was incubated for 3 hr at 37 C. A second aliquot of enzyme (1 µg) was then added and incubated for 5 hr at 37 C. The reaction was stopped by the addition of 60 µl HPLC grade glacial acetic acid and the entire reaction volume was subjected to reverse phase HPLC fraction.

HPLC Fractionation. The reaction mixture from Arg-C endoproteinase digest was injected into a 7 mm X 250 mm C18 reverse phase column (Vydak Corp.) on a Beckman Gold HPLC system. Fragments were eluted by a 0-80% gradient of acetonitrile (containing o.1% TFA, v/v) at a rate of 1% change per minute. Initial conditions utilized nanopure water containing 0.1% TFA. An enzyme blank (containing all reactants except PLA2) was run to facilitate identification of novel digest peaks. Fractions (0.5 min) were collected by a Gilson 203 fraction collector.

Electroblotting of CNBr 8 kD band. A portion of the CNBr digest-generated fragments were subjected to electroblotting to PVDF membrane for direct sequencing. 20 µl of reactants in Laemmli buffer were placed in sample well of a Novex pre-cast 16% acrylamide tricine gel and electrophoresed as in B. Following electrophoresis, the gel was placed on two thicknesses of Milligen Immobilon P PVDF membrane and electroblotted by the method of Matsudiara (1987). Transfer was accomplished at ~80 V and ~200 mA for 30 min. The membranes were briefly rinsed in nanopure water, stained in 0.1% Coomassie blue for five min, destained and air-dried. The bands corresponding to the 8 kD fragment were excised from 4 lanes and stored frozen in an eppendorf until sequenced.

Sequencing of 8 kD band. The 8 kD fragment from CNBr digest which was electroblotted to PVDF was directly sequenced on an ABI Model 473A protein sequencer at the Macromolecular Resources facility at CSU. The Arg-C endoproteinase digest fragments (HPLC-purified) were similarly sequenced after immobilzation on PVDF discs.

Raman spectrum of native PLA2. PLA2 was subjected to analysis on a Spex Raman spectrophotometer at 514.5 nm line and a power setting of 100 watts. Two milligrams of the native PLA2 were placed in a sample cup and data were collected between 475-1800 cm^{-1}. Spectra were based on summation of 12 runs recorded on a Spex Ramalog computer.

Replacement of calcium of PLA2: Activity assays. Approximately 2.7 mg of native PLA2 was dissolved in 5 ml of 1 mM EGTA and incubated at room temperature for 5 hr to remove boud calcium. PLA2 was then divided into three lots and dialyzed vs 2 x 500 ml water overnight. Samples were then either lyophilized (0-PLA2), dialyzed vs 500 ml 10 mM $CoCl_2$ (Co-PLA2) or dialyzed vs 500 ml 10 mM $CaCl_2$ (Ca-PLA2). Following dialysis, samples were desalted on a BioGel P-10 column to remove excess Ca^{2+} or Co^{2+}.

PLA2 activity was then assayed by the method of Wells and Hanahan (1969). Activities were compared with a sample of PLA2 which had not been subjected to EGTA treatment. All controls contained the appropriate concentrations of calcium or cobalt.

Purification of the Lapemis 9 kD Protein. Water-soluble proteins were extracted from a dried gland homogenate obtained from sea snakes captured in Thailand in 1989. This material was the source of crude venom. Venom was first fractionated on Sephadex G-50-50 in 10 mM phosphate buffer containing 100mM NaCl. The second peak, containing the 9 kD protein and phospholipase, was desalted, lyophilized and applied to a DEAE Sephacryl column. A linear gradient of 0-400 mM NaCl was employed to elute bound proteins. The very small yield of 9 kD protein was judged homogeneous by SDS-PAGE.

Amino Acid Composition of 9 kD Protein. Two 10 μg aliquots of the 9 kD protein were dissolved in 6N HCl and were subjected to acid hydrolysis for 24 hours. The hydrolysate was injected directly onto a Waters reversed phase HPLC (C_{18}; 10 x 300 mm) optimized for amino acid analysis and composition was determined from standardized hydrolysates.

Reduction and Alkylation of 9 kD Protein. Approximately 100 μg of 9 kD protein were dissolved in 200 μl 100 mM Na phosphate (pH 7.5) containing 1% SDS. A 50 M excess of crystal DTT was added and the sample was boiled for 3 min. After overlaying with argon, the sample was allowed to react for 2 hr at room temperature (20 C). At this time a 200 M excess of 4-vinylpyridine was added (from 0.1 M stock), overlain with argon and the solution was incubated overnight at room temperature. After dialysis against 5 mM ammonium bicarbonate (containing 0.01% SDS), the sample was lyophilized. Excess SDS was removed by extraction with acetone, TEA, acetic acid and water (85:5:5:1, v/v). Samples treated in this fashion were then used for sequencing attempts.

Deblocking 9 kD Protein With Pyroglutamate Aminopeptidase. Approximately 100 μg of 9 kD protein was dissolved in 370 μl 100 mM sodium phosphate buffer, pH 8.0, containing 10 mM EDTA, 5 mM DTE and 5% (v/v) glycerol. 12.5 μg of sequencing grade pyroglutamate aminopeptidase in 25 μl distilled water was then added and the mixture was incubated for 6 hr at 41 C. The sample was then dialyzed vs water, frozen and lyophilized.

Electrophoretic Blot of Aminopeptidase-treated 9 kD Protein. The lyophilized sample from the above deblocking step was dissolved in a tricine/reducing buffer and electrophoresed on a Novex precast 16% acrylamide tricine gel for 3 hr at ~100 volts. The 9 kD protein was thus separated from the peptidase. Following electrophoresis, the separated proteins were electrophoretically transferred to ABI ProBlot PVDF membrane using the method of Matsudaira (1987). Transfer was accomplished at 100 volts for 35 minutes. The membrane was then briefly rinsed in nanopure water, stained in 0.1% Coomassie blue, destained and air-dried. Bands from several lanes which corresponded to the 9 kD protein were carefully excised and subjected to N-terminal sequencing attempts.

N-Terminal Sequencing of 9 kD Protein. Approximately 20 μg of 9 kD protein

were immobilized on a Polybrene disc and subjected to N-terminal sequencing on an Applied Biosystems Model 473A protein sequencer. No sequence information was obtained, indicating that the N-terminus was blocked. Since snake venom toxins may contain pyroglutamate, which blocks N-terminus sequencing, pyroglutamate aminopeptidase (Boehringer-Mannheim) was used in an attempt to deblock the protein (detailed below). The first attempt was unsuccessful, and a second attempt, followed by gel electrophoresis and electroblotting of the treated protein, also failed to provide sequence information. It was concluded that the N-terminus was blocked by a residue other than pyroglutamate.

Fragmentation of 9 kD Protein With Immobilized Trypsin. 105 µl of TPCK-treated immobilized trypsin (Pierce) was washed 2X with 500 µl 100 mM ammonium bicarbonate buffer, pH 8.2. Centrifugation briefly at 6000 rpm pelleted the agarose beads containing immobilized trypsin. 130 µg of reduced and alkylated 9 kD protein was added to the gel in a total volume of 250 µl of bicarbonate buffer. A control digest containing only trypsin was run concurrently. The sample was incubated for 16 hr at 37 C, centrifuged at 10000 rpm to pellet the immobilized trypsin and the supernatant was removed and lyophilized.

Purification of Tryptic Peptides of 9 kD Protein. Peptides generated by the trypsin digest were purified by reversed phase HPLC using a Beckman Systems Gold software and a Vydak (5 µm bead, 300 nm pore) C18 column (5 mm x 300 mm). The entire sample from the above digest was dissolved in 100 µl nanopure water and injected into the column at a flow rate of 1 ml/min. After 5 min, a linear gradient of 1-80% B was initiated (A= water containing 0.1% TFA; B= acetonitrile containing 0.1% TFA); the gradient was 1%/min. Fractions were collected at 0.5 min intervals with a Gilson 203 fraction collector. Each peak was then rechromatographed on the same column with the same aqueous phase at a shallower gradient. Fractions considered homogeneous were subjected to N-terminal sequencing as above.

Identity of 9 kD Protein From Sequence Homology. In an attempt to identify the nature of the 9 kD protein, sequences of tryptic peptides generated as above were entered into the Molecular Biology Information Resources database at Baylor College of Medicine. This database provides a similarity search of all available protein sequences and identifies regions of homology. Several of the tryptic peptides showed considerable homology with sequenced proteins and the nature of the 9 kD protein has been identified.

Effect of the 9 kD protein on PLA_2. Phospholipase A_2 purified from Lapemis hardwickii venom was assayed by the method of Wells and Hanahan (1969). To determine whether or not the 9 kD protein has any modulating effect on PLA_2 activity, equimolar amounts of the two proteins were combined and assayed by the same method. Activity of the combined preparation was expressed as percent of control.

Purification of Gila Toxin. Purification of Gila toxin from (H. horridum) was modified from Hendon and Tu (8). Crude H. horridum (purchased from Miami Serpentarium) (0.5 gm) was dissolved in 0.05 M Tris-HCl, pH 8.5 containing 0.1 M NaCl and the insoluble material was removed by centrifugation (5,000 g) for 15 min at 4 C. The supernatant was then applied to a sephadex G-75 superfine column (2.5x90 cm), and separated by molecular seive chromatography with a 10 ml/h flow rate, collecting 3 ml fraction. The eluant flow was monitored by a Beckman DB-G spectrophotometer at 280 nm. The toxic fraction (fraction 2) was pooled, dialyzed against water overnight, and lyophilized. The lyophilized sample was dissolved in 3 ml of 0.05 M Tris-HCl, pH 7.5 containing 0.01 M NaCl and applied to a DEAE-sephadex ion exchange column (2.5x30 cm) previously equilibrated with the same buffer. Separation was effected by using a 600 ml 0.5 M NaCl linear gradient with flow rate 12 ml/h and collecting 3 ml fraction. The toxic fraction (fraction 4) was pooled and dialyzed against water overnight before lyophilized. The sample was dissolved in 3 ml of 0.05 M Tris-HCl, pH 7.5 containing 0.01 M NaCl and applied to QAE ion exchange column (2.5x10 cm). The toxin was eluted out by using 300 ml of 0.5 M NaCl linear gradient with flow rate 12 ml/hr. The sample was pool and dialyzed against water for 18 hr before lyophilized. The purity of toxin was shown by using SDS-PAGE base on Laemmli (1970).

pI Determination. Gila toxin was applied to a Phast isoelectric focusing gel (3-9) and focused. Phast IEF Markers were used to calibrate the gel. The

gel was fixed, washed, and then stained with Coomassie blue, then destained until the background was clear.

Amino Acid Composition. The amino acid composition was done on the non-reduced and non-alkylated G4 or Gila toxin. The protein was hydrolyzed with 6 N HCl for 24 h at 110 °C. The hydrolyzate was evaporated to dryness and the PTC derivatization method of amino acid analysis was applied. The prederivatized amino acids were then analyzed on a reverse-phase HPLC and peak areas of the unknown was compared to the amino acid standards. Detection was at 254 nm.

Carbohydrate Composition. A sample of Gila toxin was submitted for carbohydrate monosaccharide compositional analysis. The sample was sent to Oxford Glycosystems for analysis and the summary of the results are presented in the results section.

Hemorrhagic Activity. Hemorrhagic activity was assayed by method of Bjarnason and Tu (1978) with 18-22 g Swiss-Webster mice. Protein was injected in 100 ul of 0.9% saline by a subcutaneous route. After 6 hr, the mice were skinned, and hemorrhagic activity was be noted.

Toxicity. Biological assays were carried out with white Swiss-Webster mice of 18-22 g weights. Gila Toxin was injected at various dosages and routes. A constant 100 ul volume with upto 7.5 ug/g dosage was used for intravenous injections and observed for 24 hr.

Assay for enzyme substrate specificity. Arginine ester hydrolase activity was assayed using benzoly-L-arginine ethyl ester (BAEE), tosyl-L-arginine methyl ester (TAME) and N-acetyl-L-tyrosine ethyl ester (ATEE) as substrates as described by the method of Robert (1958).

Chromogenic substrates (Val-Leu-Arg-pNA, Ile-Pro-Lys-pNA, Phe-Val-Arg-pNA, Val-Leu-Lys-pNA, Ile-Glu-Gly-Arg-pNA) were assayed under the same conditions. The substrates (1 mg) were dissolved in 20 μl of DMSO and brought up to 2 ml with 0.1 M HEPES, pH 8.0, 0.1 M NaCl. The reaction mixture containing 600 μl of 0.1 M HEPES, pH 8.0, 0.1 M NaCl, 150 μl of substrate and 50 μl of toxin, was incubated at 37°C for 15 min. The reaction was stopped by adding 75 μl of acetic acid before measuring the absorbance of the samples at 405 nm.

Degradation of Fibrinogen. Fibrinogenolytic activity was measured by incubating 2% human fibrinogen solution in 5 mM imidazole-saline (1:9), pH 7.4 with 50 μg of toxin. At various time intervals, 80 μl of the incubation mixture was withdrawn and added to 80 μl of denaturing solution (10 M urea, 4% SDS, 4% B-mercaptoethanol). The samples were reduced and denatured overnight at room temperature before being electrophoresed on SDS-PAGE (Willis & Tu, 1988).

Degradation of HMW (high molecular weight) kininogen. The degradation of HMW kininogen was measured by incubating 50 μl of 2mg/ml HMW kininogen in 0.1 M Tris-HCl, pH 8.0 with 10 μl of 2mg/ml toxin at 37°C. At various times, 12 μl of the incubation mixture was withdrawn and added to 12 μl of denaturing solution (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% B-mercaptoethanol). The samples were boiled for 5 min before being electrophoresed on SDS-PAGE.

Detection of Bradykinin release from HMW kininogen. Bradykinin release was measured by incubating 30 μl of 2mg/ml HMW kininogen in 0.1 M Tris-HCl, pH 8.0 with 5 μl of 2mg/ml toxin at 37°C for 2 hr. The reaction mixture was diluted to 200 μl with 0.1 M Tris-HCl, pH 8.0 before being filtered by microcentrifugation filter with molecular weight cut off of 10,000. The filtrate was analyzed by HPLC on a vydac 4.5x25 cm C_{18} RP column. The sample was eluted with an 0-50% acetonitrile linear gradient, monitored at 214 nm. The peptide was collected and sent to MRF (Molecular Research Facility) for N terminal amino acid sequencing.

Cleavage of Angiontensin I. Cleavage of Angiotensin I was measured by incubating 100 μl of 1 mg/ml of Angiotensin I in 0.1 M Tris-HCl, pH 7.5 with 10 ul of 2mg/ml of toxin at 37°C. At various time intervals, 25 μl of reaction mixture was withdrawn and diluted to 200 μl of 0.1 M Tris-HCl, pH 7.5 before being filtered with a microcentrifugation filter with a molecular weight cut off of 10,000. The filtrate was analyzed by using HPLC on a Beckman 4.5x25 cm C_8 RP column. The sample was eluted with 0-30% acetonitrile linear gradient monitored at 214 and 280 nm and sent to MRF for N terminal amino acid sequencing.

Sequence Methods. 1. Reduction and Alkylation. Reduction and alkylation was carried out by dissolving 0.3 mg of protein in 1 ml of 0.1 M Tris-HCl pH 7.5, containing 1% SDS and 2.46 mg (15.9 μmol) DTT. The reaction mixture was

incubated at 37°C for 3 hr. The sample was alkylated by adding 6.6 mg (63.6 μmol) of 4-viny pyridine and incubated at 37°C for 3 hr before being dialyzed against 50 mM ammonium bicarbonate, pH 7.5, containing 0.001% SDS for 24 hr. 2. Cleavage of Protein. 2.1 Reaction with Cyanogen Bromide. The method was modified from Chen et al (11). The reduced and alkylated toxin was dissolved in 300 μl of solution containing 70% TFA and 30 mg/ml CNBr. The ampule was sealed and wrapped with aluminum foil before incubation for 24 hr at room temperature. The reaction was stopped by adding 10 volumes of water and was lyophilized. This step was repeated 3 times. The CNBr-cleaved toxin was run on 16% Tricine SDS-PAGE before electrotransfer to Immobilon-P membrane. The membrane was stained with 0.1% commassie blue, 10% acetic acid and 50% methanol for 1 min before destaining with 50% methanol for 5 min. The stained bands were excised and sent to MRF (Molecular Research Facility) for N terminal amino acid sequencing. 2.2 Reaction with BNPS-skatol The remaining SDS in reduced and alkylated toxin (300 μg) was extracted in a solution containing anhydrous acetone:acetic acid:triethylamine:H_2O (85:5:5:1). After centrifugation for 10 min at 5,000 rpm, the supernatant was removed. The sample was dried under the vacumn and was dissolved in 150 μl of solution containing 80% acetic acid, 2.4 mg BNPS-skatol. The ampule was sealed and wrapped with aluminum foil before incubated at room temperature for 72 hr. The reaction was stopped by adding 10 fold volume of distilled water. The excess BNPS-skatol in the sample was removed by extraction 3 times with ether at the same sample volume before lyophilized. The BNPS-skatol cleaved toxin was separated by SDS-PAGE and electrotransfered to Immobilon-P as previously described. 2.3 Reaction with Arg C Endoprotease. Remaining SDS in reduced and alkylated sample (300 μg)was removed as previously described. The sample was suspended in 100 μl of 0.1 M ammonium bicarbonate, pH 7.6 containg 0.01 M $CaCl_2$, 50 mM DTT, 5 mM EDTA and was incubated with 5 μg of Arg-C Endopeptidase (1:60 by weight) at 37°C for 18-24 hr. 2.4 Reaction with Glu-C Endopeptidase. Reduced and alkylated toxin (300 μg) was suspended in 100 μl of 50 mM ammonium bicarbonate, pH 7.8 before incubation with 8.3 μg of Glu-C Endopeptidase enzyme (1:36 by weight) at 37°C for 18-24 hr.

N-Terminal Sequencing. The gila toxin protein and protein fragments from the above cleavage methods were immobilized on a Polybrene disc and subjected to N-terminal sequencing on an Applied Biosystems Model 473A protein sequencer.

Section 3.

Results

Part I. Structure Function of Sea Snake Neurotoxin

Synthetic Peptides. The 9-fluorenylmethoxycarbamoyl (Fmoc) synthetic peptides were synthesized by the Fmoc solid phase synthesis method. The composition of the synthetic peptides were based on the amino acid sequence of Lapemis neurotoxin, following the one letter amino acid designations (Table 3-1 and Table 3-2). The nonsense peptide was synthesized with the arbitrary sequence which used all twenty common amino acids. This peptide was de .gned to serve as a negative control in all further toxicity and binding studies of the other synthesized peptide fragments of the neurotoxin. The use of all the twenty amino acids also served to check the calibra-tion and identification of the deblocked amino acids during sequencing. The synthetic peptides were cleaved from the solid phase resin and deprotected with variable yields. From 1 gm of resin treated, approximately 100 mg of crude peptide was recovered. The peptides were then subjected to further analysis and purification by HPLC. The purifications of the synthetic peptides were ac-complished using the Beckman Reverse Phase C_{18} column with an increasing linear gradient of acetonitrile described in detail in the materials and experimental section. All the peptides showed a large predominant peak for each analysis andwere easily purified by applying large amounts and collect-ing the major peaks with yields of approximately 20-26% of purified peptide with 26 mg of purified peptide B1 from 102 mg total. The other peptides showed similar yields. The major peak of each chromatogram was collected. Detection was at 214 nm. Peak elution times appear in min. (A) Peptide A1 eluted in 23.44 min.; (B) Peptide NS eluted in 38.77 min.; (C) Peptide C1 eluted in 37.87 min.; (D) Peptide B2 eluted in 33.12 min.; and (E) Peptide Loop B1 eluted at RT of 17.71 min.

Sequences of the Peptides. The synthesized peptides were subjected to sequence analysis to verify the correct syn-thesis. The results confirmed that each peptide had been correctly synthesized and each amino acid had been deprotected and not modified (including the critical tryptophan) by the synthesis and purification methods. Table 3-3 shows the results of the sequence analysis. All the peptides indicated correct synthesis, deprotection, and purity. Only the cysteine residues were not detected and shown as an X in the table. To detect cysteine residues the samples are typically carboxymethylated and detected as the CM-cys PTH derivative.

Sea snakes (*Lapemis hardwickii*) were obtained from local fishermen in Songkhla, Thailand. A total of 470 g powdered glands was obtained from the sea snake dried glands for venom extraction.

After the extraction procedure, approximately 46.5 g of gland extract (crude venom) were obtained from 470 g dried glands or approximately 10% yield by weight. The approximate LD_{50} of the crude venom was 9.0 μg/g, which is similar to values obtained for earlier venom extractions. Crude venom has a relatively low toxicity due to "dilution" with nontoxic intracellular proteins.

Step I, Sephadex G5-50 Gel Filtration: The fractionation pattern for the crude venom yielded eight peaks. Peak I and Peak II contains most protein and enzyme components with a Mr > 20,000 daltons. Peak III contained mainly two proteins with Mr of approximately 15,000 and 10,000 daltons; the former is likely a phospholipase A2 while the latter may be the neurotoxin precursor. Peak IV contained the major neurotoxin (NT) of *Lapemis* venom. Peaks V-VIII are peptide components of the venom.

Step II, CM-Sephadex C25 Ion Exchange chromatograph: The elution pattern used the pooled toxic peak IV recovered from step I. Two peaks were seen, one before the gradient was applied and one after the gradient was applied. The two peak fractions were recovered termed IV.1 and IV.2. The IV.2 was the Lapemis toxin based on toxicity screen test with mice and SDS-PAGE.

The venom from *Naja naja atra* was subjected to Sephadex G50-50 gel filtration pre-equilibrated with 10 mM sodium phosphate buffer (pH 6.5) containing 0.1 M NaCl. The peak containing the neurotoxin (peak IV) was pooled, dialyzed and lyophilized. This lyophilized fraction was then subjected to CM-Cellulose or CM-Sephadex C25 ion exchange chromatography pre-equilibrated with

10 mM sodium phosphate buffer (pH 7.8). After one column volume of buffer had been eluted a linear salt gradient from 0 M NaCl to 1 M NaCl (in 800 ml) was applied. After the gradient was applied, the first major peak was determined to be the neurotoxin after gel electrophoresis, toxicity checks and amino acid composition (Table 3-4). The isolation of cobrotoxin was done repeatedly to generate a store of this neurotoxin to be used in the isolation of the AChR.

The homogeneity of the Lapemis toxin and cobrotoxin was established by two independent methods, PAGE and analytical HPLC using a Beckman ultraphere-ODS column (4.6 mm x 25 cm). A single band was observed on acrylamide gels after applying Lapemis toxin or cobrotoxin. The HPLC chromatography pattern also showed that only one protein was present for each toxin. The LD_{50} of the Lapemis neurotoxin was similar to the value reported previously (Tu and Hong, 1971) of 0.065 $\mu g/g$ and the 0.07 $\mu g/g$ for cobrotoxin (Yang, 1965).

Isolation of Lapemis toxin was done repeatedly to generate a store of the neurotoxin. Aliquots of 10 μg were made, lyophilized and stored at -20 °C to be used later in the binding studies. Isolation of cobrotoxin was done repeatedly to generate a store of the neurotoxin which was stored lyophilized at -20°C until used in the affinity chromatography purification of the acetylcholine receptor.

Isolations of AChR yielded solutions of approximately 0.2 to 0.3 mg/ml protein concentration based on the BCA protein assays. The results of the last acetylcholine receptor isolation gave approximately 25 ml of 0.27 mg/ml solution. The SDS-PAGE showed the expected four homologous subunit bands of the receptor with a slight contamination of two weak higher molecular weight bands. Using 270,000 g/mol as the receptor molecular weight gave a receptor solution concentration of 1×10^{-6} M.

Determination of the Equilibrium Dissociation Constants.

[125-I] Radiolabelling of Lapemis toxin (LTX), tyrosine containing synthetic peptides and α-bungarotoxin (α-Bgtx) was done using the α-bungarotoxin (10 μg), lapemis toxin (10 μg) and each peptide (5 μg to 10 μg). These toxins and peptides were labelled using the Chloramine-T method with 1 mCi of $Na^{125}I$ under optimized conditions to favor iodination of the tyrosine residue only (Hunter & Greenwood, 1962; Atassi, 1977). This resulted in a specific activity of approximately 106 and 80 $\mu Ci/pmol$ for the α-Bgtx and LTX, respectively. The peptides resulted in specific activities for peptide NS of 41.6 $\mu Ci/pmol$; peptide B1 of 36.4 $\mu Ci/pmol$; peptide B2 of 25.6 $\mu Ci/pmol$; and for peptide CA-B1 of 8.3 $\mu Ci/pmol$.

The general result of the peptide binding study indicates that peptide B1 apparently binds while the reduced and alkylated CA-peptide B1, peptide B2 and the nonsense peptide NS do not bind or weak binding was not detected. Figure 3-1 (A) is a binding curve plot of Lapemis toxin. The 50% radio-activity level gave an estimate of the equilibrium dissoc-iation constant of ? nM. Figure 3-1 (B) is a plot of the bound activity of peptide B_1 to the acetylcholine receptor versus the concentration of B_1. The bold curve indicates the activity of the peptide with receptor or total activity while the light curve indicates non-specific binding. The bold curve minus the light curve will give the specific binding and thus peptide B_1 appears to bind weakly to the receptor with an estimated K_D of 35 to 40 nM. Plots of the other tested peptides follow the non-specific binding plot and therefore appear not to bind at all. Figure 3-1 (C) is a plot of the carboxyamidomethylated B_1 peptide showing a typical plot where the total binding and non-specific binding are nearly the same indicating no detected binding to the AChR. Figure 3-1 (D) depicts the plots of the data using 1 nM AChR and without using AChR from titrating with the radiolabelled α-bungaro-toxin. Specific binding is the total binding minus the non-specific binding. Saturation occurs at about 20,000 cpm radioactivity. Therefore, approximately 10,000 cpm radio-activity would be the 50% activity level. The concentration of α-bungarotoxin at the 50% radioactivity level is the equilibrium dissociation constant (K_D). This control experiment using radiolabelled α-Bgtx resulted in a K_D of approximately 1 nM as seen by the concentration of ligand at 50% of the saturation level which is the same as the published value for this assay method.

From a plot of bound vs free, the K_D may be estimated by the ligand concentration at 50% of the maximum (saturation) bound activity. The plot of

bound vs total is experimentally the same as bound vs free since the bound is much less than free, thus free is approximately the same as the total ligand concentration (Bennett, Jr., 1977).

The central core portion (peptide B1) (see Table 3-1) of the toxin does retain binding ability although about one order of magnitude less strongly than Lapemis toxin or α-bungarotoxin. Also, Lapemis toxin is only slightly weaker binding (2 nM) than the long chain α-bungarotoxin (1 nM). Lapemis toxin was previously shown to inhibit [125-I]α-Bgt binding to the AChR. (Allen and Tu, 1985).

The fact that peptide B2 and the CA-B1 peptide had no detectable binding suggests the importance of the intramolecular disulfide bond of peptide B1 and its structural need in the binding ability of the peptide and presumably in the intact neurotoxin. Raman spectroscopy of peptide B1 indicated no free sulfhydryl bands (absence of bands in the 2578 cm^{-1} region and that the disulfide bond existed in a trans-guache-guache conformation with band at 525 cm^{-1}. In the intact neurotoxin the disulfide bond stretching vibrations occur at 512 cm^{-1} and assigned to the gauche-gauche-gauche conformation. The single free sulfhydryl group of the toxin is readily detected at 2578 cm^{-1} (Fox et al., 1977).

The Ellman's reagent test during the carboxyamido-methylation confirmed the absence of a free sulfhydry group and presence of disulfide bridges. The result of the sizing Sephadex G-25 column during radio-labelling determined the peptide to be most likely in the monomer form.

Toxicity of the Peptides. All tested mice survived the intravenous injections of the peptides with no apparent ill affects. The amount of sample injected (8 μg/g) is significantly higher (approx. 114 X) than the LD_{50} dosage of 0.06 - 0.07 μg/g of purified neurotoxin indicating the synthetic peptides are relatively non-toxic. Peptide B1 was further tested on three additional mice at the same dosage. The animals all survived with no apparent ill affects. At necropsy, these mice all appeared normal upon gross observation of the viceral organs when compared to two normal control mice. The nonsense peptide was also made and used to serve as a negative control to eliminate any artifacts that may be introduced by the method of synthesis and preparation. Table 3-5 summarizes the results of the binding and toxicity studies.

Hydrophilicity Analysis. The hydrophilicity analysis of Lapemis toxin indicated that the toxin is predominately hydrophilic with the loop B region (residues 22-39) having the highest average hydrophilic values for the moving hexapeptide window through the linear amino acid sequence. It should be noted that loop A (residues 3-17) and loop C (residues 41-52) are also relatively hydrophilic regions (Figure 3-2).

When this information is combined with the fact that the loop B1 retains some binding ability plus lacks toxic effects at relatively high dosages and combined with the correlation of high antigenicity of hydrophilic regions of proteins to produce specific antibodies suggests that loop B1 fragment (and perhaps loops A and C fragments) should be tested as an <u>in-vivo</u> antagonist of neurotoxins and should be tested for uses as a specific antivenin agent or vaccine applications.

Venom Extraction from Sea Snake Collection.

Sea snakes (<u>Lapemis hardwickii</u>) were obtained from local fishermen in Songkhla, Thailand. Glands were dissected out and dried in a cool room. Dried glands were then pulverized to a coarse powder using a Wiley mill. A total of 470 g powdered glands was obtained for venom extraction.

Cold glass-distilled water was added to approximately 20 g dried gland powder, and the resulting paste was ground (using a mortar and pestle) for 40 min at 4 °C. The paste was then suspended in approximately 600 ml cold distilled water, stirred vigorously and allowed to sit for 10 min at 0 °C. The suspension was then stirred, divided into two 400 ml centrifuge bottles and was centrifuged for 20 min at 4000 rpm (4 °C). The supernatant was collected, shell-frozen and lyophilized, and the remaining solids were ground and extracted again (as above). Approximately 46.5 g of gland extract (hereafter referred to as crude venom) were obtained from 470 g dried glands.

All isolations closely followed the previously published methods of Tu and Hong (1971), Tu et al. (1975) and Mori and Tu (1988). To compare venoms

collected in 1986 and 1989, 240 mg samples of each were subjected to gel filtration on Sephadex G-50-50 using 10 mM sodium phosphate buffer (pH 6.5) containing 0.1 M NaCl. Samples were dissolved in 3.5 ml buffer, briefly centrifuged to remove insolubles and applied to a 2.5 cm X 100 cm Sephadex column at 4 °C. Flow rate was 12 ml/hr, and fractions were collected at 15 min intervals. Absorbance at 280 nm was used to estimate protein and peptide concentration.

Toxicity of crude venom was evaluated using female Swiss/Webster mice. Crude venom was dissolved in 0.9% saline at various concentrations and 100 µl was injected IV (tail vein). All doses were adjusted to individual body weights. LD50 values (24 hr) were estimated from semi-log plots of survivorship curves.

Comparison of 1989 and 1986 Lapemis hardwickii Venom Samples

Toxicity: The estimated LD50 for 1989 crude venom is 9.0 µg/g, similar to values obtained for earlier venom extractions. Crude venom has a relatively low toxicity due to "dilution" with nontoxic intracellular proteins.

Gel Filtration: Fractionation patterns for 1986 and 1989 crude venoms are similar (not shown). Patterns for the two batches of venom were qualitatively very similar. Peak I contains most protein and enzyme components with a Mr > 20,000 daltons. Peak II contains two proteins with Mr of approximately 15,000 and 10,000 daltons; the former is likely a phospholipase A2 while the latter may be the neurotoxin precursor. Peak III contained the major neurotoxin (NT) of Lapemis venom. Peaks IV-VIII are peptide components of the venom.

The fractionation patterns were also quite similar quantitatively. Some differences exist in the amounts of Peak II, the phospholipase and the putative neurotoxin precursor, and of peptide Peak IV; both were larger in the 1989 sample. However, these differences are minor, and the two batches of venom can be considered essentially the same.

The Step II CM-Sephadex C50 Ion Exchange chromatograph pattern using the toxin (8 mg) recovered from step I peak III (Figure not shown) indicates two peak fractions were recovered termed III.1 and III.2. The III.2 is the Lapemis toxin based on toxicity screen test with mice and SDS-PAGE with 480 µg recovered.

Isolation of cobrotoxin.

The venom from Naja naja atra was subjected to Sephadex G50-50 gel filtration pre-equilibrated with 10 mM sodium phosphate buffer (pH 6.5) containing 0.1 M NaCl. The third peak containing the neurotoxin was pooled, dialyzed and lyophilized. This lyophilized fraction was then subjected to CM-Cellulose ion exchange chromatography pre-equilibrated with 10 mM sodium phosphate buffer (pH 7.8). After one column volume of buffer had been eluted a linear salt gradient from 0 M NaCl to 1 M NaCl (in 800 ml) was applied. The first major peak after the gradient was applied was determined to be the neurotoxin after gel electrophoresis and toxicity checks. This method is essentially that of Tu and Hong (1971).

Isolation of the Torpedo californica Nicotinic Acetylcholine Receptor.

The isolated cobrotoxin from Naja naja atra cobra venom was used to make an affinity column using CNBr-activated Sepharose 4B resin. The T. californica electroplax tissue was homogenized in Torpedo Ringers Isolation buffer which included various protease inhibitors; the homogenate was filtered through several layers of cheesecloth and then passed through the affinity column. The cobrotoxin specifically binds the AChR allowing all remaining material to be eluted first. The AChR is then eluted using the competitive inhibitor carbamylcholine. The purified solubilized receptor is then used in the AChR Binding Assay. The methods followed were that of Froehner and Rafto (1979) or Linstrom et al. 1980.

Purity of Toxin. The homogeneity of the Lapemis toxin was established by two independent methods, PAGE and analytical HPLC using a Beckman ultraphere-ODS column (4.6 mm x 25 cm). A single band was observed on acrylamide gels after applying 10 µg of Lapemis toxin. The HPLC chromatography pattern also showed that only one protein was present. The LD_{50} of the neurotoxin was similar to the value reported previously (Tu and Hong, 1971)

Free Lapemis Toxin. Before the chemical modification of AChR-bound neurotoxin, free toxin was used as a control. In order to find the optimum reaction time, the effect of reaction time for the degree of arginine

modification as detected by cpm was first investigated. As the phenyl [2-^{14}C]glyoxal was added, the toxin was modified, as can be seen from the increased specific activity in neurotoxin, and it reached the saturation point in 180 min. Because the arginine modification by phenyl [2-^{14}C]glyoxal required 3 h, we used the reaction time of 3 h for all modifications. The modification of the arginine residues in free neurotoxin was evidenced from the large amount of ^{14}C found in the toxin after incubation with phenyl [2-^{14}C]glyoxal. In experiment 1, 1688 cpm was found, and in Experiment 2, 1501 cpm was found. From amino acid analysis it was evident that 2 out of 3 mol were modified.

The next important step was to identify which of the three arginine residues in the neurotoxin were modified. In order to do this, the modified toxin was incubated with endoproteinase glutamine C. The proteolytically digested fragments were separated into four components by HPLC using a C_{18} reverse-phase column. The amino acid composition of each fragment was done.

Since the amino acid sequence of Lapemis toxin is known, the cleavage sites can be determined. Fragment 3 showed 1 mol of arginine, but fragment 4 contained no arginine. Thus Arg-31 and Arg-34 were modified, while Arg-37 was not modified. Apparently Arg-37 was unavailable for modification because it was situated in a more interior part of the toxin. The modified toxin was nontoxic to mice at 0.5 μg/g concentration, while the LD_{50} of unmodified toxin was 0.06 μg/g.

AChR-Bound Lapemis Toxin. Lapemis toxin was mixed with AChR with a molar ratio of 2:1. When the complex was formed, the neurotoxin peak that normally would appear at tube 95 on Sephadex G-50 chromatography disappeared.

The receptor-bound toxin was modified with phenyl [2-^{14}C]glyoxal at 100-fold excess. When phenyl[2-^{14}C]glyoxal was used to modify arginine residues, a very large amount of ^{14}C was incorporated. In two separate experiments, the ^{14}C incorporated was 3756 and 3793 cpm, more than double the amount (1688 and 1501 cpm) incorporated into free neurotoxin. This suggests that many arginine residues in the AChR of the AChR-Lapemis toxin complex were modified by this reagent.

For our objective, it was most important to determine how many arginine residues of AChR-bound neurotoxin were modified. For this purpose the Lapemis toxin was detached from the AChR-Lapemis toxin complex and the radioactivity of the detached neurotoxin was determined. The receptor was removed by precipitation after incubating the complex in NaCl at 37 °C for 6 h. After removing Triton X-100, the lapemis toxin was purified by HPLC. Amino acid composition analysis of the HPLC peak indicated that the fraction was indeed Lapemis toxin.

The Lapemis toxin detached from the AChR-neurotoxin complex had and amino acid composition identical to that of unmodified toxin, yet only 176-185 cpm, close to background radioactivity, were found in the two experiments. This indicates that none of the arginine residues of AChR-bound Lapemis toxin were modified by phenyl[2-^{14}C]glyoxal. Presumably the two arginine residues that are available form modification in the free toxin are no longer accessible to modification because these two residues are involved in the binding to AChR.

The results of chemical modification of neurotoxin before and after binding to AChR are summarized as follows:

	Free Lapemis toxin	AChR-bound Lapemis toxin
Arg 31	modified	unmodified
Arg 34	modified	unmodified
Arg 37	unmodified	unmodified

Isolation and Search for Lapemis Toxin Precursor

Fractionation of crude Lapemis gland extract on Sephadex G-50 produces a highly repeatable pattern of 8-9 peaks (Figure 3-3A). Peak I contains most cellular components larger than ~15-20,000 daltons. Peak II consists of 2 proteins of ~13,500 and 9000 daltons. Lapemis neurotoxin content of this preparation is quite low and elutes as a broad third peak. Peaks IV-IX are peptides of less than 2000 daltons and other UV-absorbing compounds.

Peak II contains proteins which could be neurotoxin precursors, and these fractions were subjected to ion exchange. The 9000 dalton protein is apparently a minor component and eluted as peak II (Figure 3-3B). The major peak (IV) contained the the 13,500 dalton protein. Since the yield of this protein was highest, it was decided to characterize this fraction further. Peak IV was chromatographed on BioGel P-10 to remove a small amount of higher molecular weight contaminant (Figure 3-3C). From SDS-PAGE, this protein appears to be homogeneous and has a molecular weight of 13,500 daltons. It is an acidic protein and has an apparent pI of 4.6.

The molecular weight of the 13.5 kD protein is somewhat high for a potential neurotoxin precursor, but it is in the correct range for the common venom enzyme phospholipase A2. Subsequent assays confirmed that this protein is indeed a phospholipase A2. When incubated with phosphatidylcholine (dipalmitoyl) substrate, the enzyme has a specific activity of 62.7 μmol fatty acid released/mg protein/minute.

Approximately 7.0 mg of pure phospholipase was obtained from 1.0 g crude Lapemis gland extract. This material gave a single band at ~13,500 following SDS-PAGE and has an isoelectric point of 4.6. All following results were obtained from this preparation of PLA2.

Initial N-terminal sequencing of the reduced and alkylated PLA2 provided the first fifty amino acid residues (Table 3-7), but due to decreasing repetitive yields further direct sequencing was not possible. PLA2 was therefore chemically cleaved with the intent of producing shorter fragments from which to obtain further sequence information. Cyanogen bromide fragmentation occurs via the destruction of and chain hydrolysis at methionone residues; from amino acid composition analysis, Lapemis PLA2 has 3 methionine residues (Table 3-6), and therefore up to four fragments should be produced by CNBr cleavage. Initial digest characterization on SDS-PAGE revealed three fragments which could be detected by Coomassie blue staining, and there was no evidence of parent material. Hydrolysis by CNBr was therefore judged complete.

Purification of digestion fragments by reversed phase HPLC was inadequate due to retention of the large fragments by the column matrix. CNBr-generated fragments were subjected to SDS-PAGE on tricine buffered gels, which favors the separation of low molecular weight proteins/peptides as well as providing information on size of fragments. The 8 kD band is the prominant product of hydrolysis, and electroblotting to PVDF provided sufficient amounts for direct amino acid sequencing. The sequence of this fragment was obtained for the first 12 residues and is shown in Table 3-8; the N-terminal methionine was deduced by sequence homology and position. The low quantity of protein transferred to the PVDF membrane prevented further sequence determination.

To obtain further sequence information on the 8 kD fragment, the CNBr digest was subjected to SDS-PAGE and electroelution. This method allows the collection of purified proteins resulting from SDS-PAGE fractionation and is based on the Hunkapiller electroelution device. Approximately 10 μg of purified 8 kD fragment were obtained and cleavage was attempted with Arg-C endoproteinase. Three unique peaks resulted from this enzyme digest and subsequent reversed phase HPLC fractionation.

Upon sequencing, only one peak yielded sequence (38.2 min peak); this fraction has the same N-terminal sequence as the undigested 8 kD fragment. However, this preparation yielded 35 residues of sequence, and it is believed that the Arg-C endoproteinase digest removed an arg-peptide from the 8 kD fragment which is close to the C-terminal portion of the molecule. By comparing sequence with known sequences of PLA2 from Laticauda species, the 8 kD fragment is believed to begin at residue 58, and the undigested fragment may represent the entire C-terminal half of the native PLA2.

Lapemis PLA2 shares considerable sequence homology with other sea snake

PLA2 enzymes (Tables 3-7 & 3-8) as well as with most other elapid PLA2 (not shown). Several general points can be made from the portions of the molecule for which sequence has been obtained. Residues 28 (tyr), 30 (gly), 32 (gly) and 49 (asp) are highly conserved in most PLA2 and are believed to be involved in calcium binding (White et al., 1990). These residues are identical in Lapemis PLA2 and it is assumed they are also involved in calcium binding. Residue 48 (his), part of the catalytic center of the enzyme, is also present in the Lapemis enzyme. Outside these functional domains, however, there is much lower sequence homology. Even among the sea snake PLA2, the greatest region of sequence homology apparent at present occurs in the region of residues 24-50, which includes the core of the calcium binding site. Substitutions in this region may have critical results, and an inactive PLA2 homologue isolated from Laticauda colubrina venom was shown to have an asparagine residue at position 48, normally a histidine residue and part of the catalytic center.

To determine some structural features of the Lapemis PLA2, the native enzyme was subjected to Raman spectroscopy. A very prominent peak, partly obscured by fluorescence, is seen at 507 cm^{-1} and represents the S-S stretching band resulting from the prominent disulfides of this protein. Calcium is required for PLA2 activity, and the binding sites in homologous enzymes have been determined (White et al., 1990). To determine the requirement for calcium by the Lapemis PLA2, native calcium was removed by EGTA and replaced by cobalt, another divalent cation. The results of calcium removal/replacement experiments are shown in Table 3-9. After removal of endogenous calcium with EGTA, activity was reduced to 27% of controls. It is concluded that calcium is required for activity, and residual activity after treatment may result from tightly bound calcium or from potential calcium contaminants present in nanopure water. At least 90% of native activity can be recovered after EGTA treatment by the addition of 10 mM $CaCl_2$, demonstrating that there is no permanent loss of structure when calcium is removed. However, cobalt replacement nearly abolished activity, and the enzyme has only 3% of native activity in the presence of 10 mM $CoCl_2$. Inactivation may result from steric hinderance from this larger metal cofactor or perhaps the lower conductivity of the cobalt ion is incapable of supporting the catalytic network of the active site. It is likely that tertiary structure is affected by replacement with cobalt, and spectroscopic studies are currently in progress to evaluate this hypothesis.

Venom from Lapemis hardwickii yields 8 fractions when subjected to gel filtration. Peak II contains two proteins, a 13.5 kD protein (PLA_2) and a 9 kD protein (neurotoxin precursor?). When subjected to ion exchange chromatography on DEAE Sephacel, PLA_2 is obtained in good yield, but only a small quantity of the 9 kD protein is recovered (fractions 100-110). Since larger quantities of PLA_2 were obtained in pure form, it was decided to initiate sequence studies on this protein until a sufficient quantity of the 9 kD protein is accumulated.

The 9 kD protein was obtained in very low yield from crude Lapemis gland extract. A combination of gel filtration, DEAE ion exchange and a third gel filtration step gave approximately 350 μg of purified protein from 1 g of extract. This protein gave a single band at ~8800 D following SDS-PAGE and has an isoelectric point of 5.2. Several preparations of the 9 kD protein were required to perform the above modifications and sequencing runs. Results of amino acid analysis are shown in Table 3-10.

Initial N-terminal sequence attempts were unsuccessful, most likely as a result of N-terminal blockage. Since pyroglutamate residues have been found at the N-terminus of other snake toxins, it appeared likely that the 9 kD protein was similarly blocked. However, after treatment with pyroglutamate aminopeptidase, sequencing was still blocked, indicating another modified amino acid residue. To obtain sequence information which would reveal whether or not the 9 kD was a neurotoxin precursor, digestion with immobilized trypsin was performed.

The result of tryptic digestion and subsequent purification of tryptic peptides at least 16 major peptide peaks were resolved. Peptide peaks contain very little extraneous material and are suitable for sequencing.

At present, 9 tryptic peptides have been sequenced, of which two are identical. Amino acid sequences are given in Table 3-11. As expected, all

peptides terminate in lysine residues, indicating that the lysine content based on amino acid analysis is low. None of these peptides show sequence homology with Lapemis toxin or signal peptides of other sea snake toxins which were deduced from DNA sequences. The 9 kD protein therefore does not represent a neurotoxin precursor.

From sequence homology comparisons, it was found that the tryptic peptides show considerable similarity to the calcium-binding protein parvalbumin. Not surprisingly, homology is greatest with a parvalbumin which was isolated from snake muscle (Maeda et al., 1984). By aligning regions of homology of tryptic peptides with the amino acid sequence of snake parvalbumin, tentative assignment of the position of unique peptide fragments was made. This alignment is shown in Table 3-12. Of 61 residues sequenced thus far, 51 of the Lapemis parvalbumin are identical with those of the boa constrictor parvalbumin (~82%).

Parvalbumins are intracellular calcium-binding proteins found in distinct cells of muscle, brain and endocrine gland tissues (Heizmann, 1984). However, they have never been reported from venom glands or venoms of snakes. Since phospholipase A_2 is the main enzymatic component of many sea snake venoms and since it requires calcium, parvalbumin may have a modulating effect on PLA_2 activity. Based on activity assays in the absence and presence of parvalbumin, there is no effect on phospholipid hydrolysis by PLA_2. The specific role of parvalbumin in Lapemis venom remains unclear.

Purification of Gila Toxin. Figure 3-4 depicts the chromatograms of the Gila toxin isolation. The first step of the isolation used gel filtration (Sephadex G-75) chromatography and peak 2 was pooled for the second step. Step two used ion exchange chromatography (DEAE-Sephacryl) and G4 peak was pooled for the final step. Step three (final step) used ion exchange chromatography (QAE) and the single peak G4 after the salt gradient was applied was pooled and determined to be the Gila toxin.

Mr Determination of Gila toxin. From the SDS-PAGE for the Mr determination of Gila toxin with the molecular weight markers were electrophoresed (Sigma SDS-7) gave Mr of protein G3 as approximately 28,000. The protein G4 or Gila toxin protein Mr was approximately 33,000.

pI Determination. The pI of the Gila toxin isolated was approximately 4 (3.75-4.2). This determination will be repeated using a narrow range pH gradient gel to improve the accuracy of the determination.

Amino Acid Composition. Table 3-13 presents the amino acid compostional results of Gila toxin. The values listed are the minimum residues based on phenylalanine. Hendon and Tu (1981) values are presented for comparison. The analysis is being repeated with reduced and alkylated Gila toxin to determine the 1/2 cystine content and solve the lost histidine value.

Carbohydrate Composition. Table 3-14 presents the summary of the carbohydrate composition of Gila toxin. The results listed in the table are for the monosaccharide composition. The glycosylation of the carbohydrate appears to be an Asn N-linked glycosylation as there was no N-acetylglucosamine found which indicates a lack of O-linked glycosylation.

Hemorrhagic Activity. Gila toxin at the 50 μg dose did not cause any hemorrhage in the skin. Further histological studies are planned to look at major tissues. For example, the kidney, lung, liver, intestine, brain and other selected tissues.

Toxicity. Toxicity checks indicate that the isolated Gila toxin was toxic/lethal at a dosage near the LD50 of the crude venom and Gila Toxin of 2.5 ug/g (iv mice). Some animals died within 30 minutes of injection from both subcutaneous and intravenous routes of injections from dosages of 2.5 mg/kg. Tu and Hendon (1981) reported the LD_{50} OF Gila Toxin to be 2.5 mg/kg to 2.75 mg/kg. Table 3-15 compares Gila toxin and Protein G4 and G4 was concluded to be the Gila toxin isolated and characterized by Tu and Hendon (1981). Further in-vivo studies are planned to include clinical signs, necropsy, histological studies and LD_{50} determination. From these studies the functional aspects of the Gila toxin should become more apparent.

Enzymatic Assays. Gila toxin hydrolyzed N-Benzoyl-arginine-ethyl ester (BAEE) and N-Tosyl-arginine-methyl ester (TAME) with a specific activity of 245 unit/mg and 209 unit/mg respectively as indicated in Table 3-16. However Gila

toxin did not hydrolyze N-acetyl-L-tyrosine ethyl ester (ATEE) which indicates that Gila toxin is a trypsin like enzyme rather than a chymotrypsin like enzyme. The hydrolytic activity was inhibited by DFP (diisopropyl fluorophosphate) but not inhibited by EDTA which indicates that Gila toxin is a serine type protease.

Although Gila toxin is a trypsin-like enzyme, Gila toxin did not hydrolyze all of the substrates which contain arginine or lysine. Additionally, Gila toxin hydrolyzes only a specific substrate. Gila toxin hydrolyzed N-Benzoyl-Phe-Val-Arg-pNA substrate at specific activity of 51.6 unit/mg which indicates that Gila toxin is a thrombin like enzyme.

Fibrinogenolytic Activity. SDS-PAGE analysis (Figure 3-5) showed Gila toxin to be an A ,B and fibrinogenase. The A was slowly degraded after 1 hr followed by degradation of B . Both A and B were completely degraded after 6 hr. The fibrinogenase also digested chain after 12 hr of hydrolysis. The Gila toxin fibrinogenase activity was different from Crotalas atrox (snake venom) fibrinogenase. C. atrox fibrinogenase degrades the A and B chains within 1 hr, but does not have any effect on chain even after 24 hr of hydrolysis. The degradation products from C. atrox were also different from Gila toxin which indicates the different cleavage sites on fibrinogen.

Degradation of HMW (high moloecular weight) kininogen. The partial N-terminal sequence of Gila toxin (Table 3-17) shows homology to crotalase, C.atrox (EI), kallikrien, and thrombin. Kallikrien is a proteolytic enzyme which cleaves high molecular weigh kininogen (HMWK) (114 kD) at specific sites releasing bradykinin (1 kD) as shown in Figure 3-6. The degradation product light chain (58 kD) is further digested giving rise the modified light chain (45 kD) and fragment 13 (13 kD). Figure 3-7 shows the SDS-PAGE of HMW kininogen digestion by Gila toxin. The degradation product of HMW kininogen (114 kD) by Gila toxin produced the light chain (58 kD). Further cleavage on light chain also produced the modified light chain (45 kD). The degradation of HMW kininogen by Gila toxin show similarity to degradation products of plasma killikerien on SDS-PAGE.

Bradykinin release. HMW kininogen was incubated with Gila toxin at room temperature for 1 hr before being filtered by microcentrifuge filtration with a molecular weight cutoff of 10,000. The filtrate was loaded on a HPLC C_{18} reverse phase column. The peptide product was eluted with acetonitrile. As a control, HMW kininogen was incubated alone without Gila toxin as shown in Figure 3-8A. The degradation of HMW kininogen by Gila toxin (Figure 3-8B) releases a peptide with the retention time of 40 min which is the same retention time as pure bradykinin (Figure 3-8C). Five amino acid residues from the N-terminal of this peptide were sequenced and compared to bradykinin as shown in Figure 3-9.

Degradation of Angiontensin I. Angiontensin I was incubated with Gila toxin at various times before being filtrated by microcentrifuge filtration with a molecular weight cutoff of 10,000. The filtrate was loaded onto a HPLC C_8 reverse phase column. The peptides were eluted from the column by a linear gradient of 0-50% acetonitrile. Figure 3-10 shows that incubation of Angiotensin I with Gila toxin produced peptide B. The amino acid N-terminal of peptide B was sequenced and compared with angiotensin I,II,III as shown in Figure 3-11.

Primary structure of Gila toxin. The primary sequences of Gila toxin were analyzed by using the chemical and enzyme cleavages of CNBr, BNPS-skatol, Arginin C endopeptidase, and Glutamine C endopeptidase. The degradation products of each method were sequenced and the results analyzed for overlapping sequences. Figure 3-12 shows the amino acid sequences that have been analyzed.

Section 4.

Discussion

The snake postsynaptic neurotoxins are structurally compact, stable molecules that bind tightly and specifically to the alpha subunits of the acetylcholine receptor blocking nerve transmission. The study into the detailed mechanism of this binding and the related structural information is continuing using a variety of strategies and approaches in an effort to map and understand at the molecular level the neurotoxin receptor interaction.

The sequences of the subunits of the neuromuscular nicotinic acetylcholine receptor have been deduced from the cDNA sequences of the four subunits known to make up the pentamer receptor with an overall molecular weight of approximately 270,000 and acidic pI's 4.9-5.4. These subunits are designated as $_2$ for several species including the *Torpedo californica* receptor (Noda et al., 1983, Claudio et al., 1983). The minimum number of lipids required for a functional AChR was determined and reported by Jones et al. (1988). The structures of the carbohydrate moieties linked to each AChR protein subunits were elegantly determined by Nomoto et al. (1986).

The methods used in this research were developed over several years based on the fact that snake venom postsynaptic neurotoxins were discovered to bind tightly and specifically to the nicotinic acetylcholine receptor found at the neuromuscular junction (Weber & Changeux, 1974; Lentz & Wilson, 1988). The pentamer intramembrane acetylcholine receptor functions as an ion channel for the relay of the neurotransmission from the axon to the effector muscle cell by the binding of the neurotransmittor acetylcholine to the alpha subunits. The conformational change to allow the influx of cations to propagate the signal is thus accomplished. The acetylcholinesterase then hydrolyzes the acetylcholine to allow for the return to the non-depolarized state of the muscle cell and to the closed conformation of the receptor channel. This is schematically presented in figure 4-1. The postsynaptic neurotoxin binds to the receptor and blocks the opening of the ion channel thus preventing the nerve transmission. The neurotoxins have been shown to specifically inhibit the binding of acetylcholine and bind to the alpha subunit of the receptor as depicted in figure 4-2. The exact mechanism and detailed binding sites are still in doubt but should soon, with further studies, be clarified at the molecular level.

Table 4-1 summarizes the synthetic peptide binding studies to the acetylcholine receptor from this work and compares the findings to the Elapid long chain postsynaptic neurotoxin (-bungarotoxin) and a cobra short chain neurotoxin. These results support the conclusion that the central loop B region of lapemis toxin and other neurotoxins plays the dominant role in receptor binding.

The study of the conserved amino acids found in the sequence of the known short chain neurotoxins provide clues to which amino acids play some role in the binding and clues to which amino acids may not be specifically important to the structure function of the neurotoxin. Figure 4-3 depicts the conserved amino acids of the known short chain neurotoxins. From this figure, the synthetic approach or the mutagenic cloning approach can be used to selectively change and test certain amino acids or regions of the neurotoxin with respect to structure, binding and toxicity. The conserved residues, especially ones that have no known chemical modification method, could be checked by conservative and non-conservative substitution and the effects on toxicity and binding observed. These studies would further map the molecular interaction of the neurotoxin ligand binding site on the receptor.

From this data, neurotoxin analogues may be designed to antagonize the neurotoxin and its effects. These antagonists may also, with appropriate studies, prove to be useful drugs in various aspects of neuromedical science studies and treatments. Antibodies against neurotoxin analogues may be produced more effectively that may recognize and neutralize the native neurotoxin. Perhaps, vaccines may be produced to aid in neutralization of this class of neurotoxins if the neurotoxin analogues are non-toxic and immunogenic.

This synthetic approach used in this work has proved to be very effective and straight forward. Future experiments using this strategy are very feasible. Solid phase peptide synthesis (SPPS) was first introduced by R. B. Merrifield in 1963 reporting the synthesis of a tetrapeptide which was a revolutionary breakthrough (Merrifield, 1963). The last few decades of research have produced many new improvements and chemistries to synthesize peptides and proteins. In the early 1970's the complete postsynaptic neurotoxin, cobrotoxin (60 amino acids residues) was synthesized *de novo* by Wong et al. (1978) showing the native neurotoxic activity. Articles and textbooks have been written on many aspects of peptides synthesis (eg. Kent, 1988; Paivinen et al., 1987; Bodansky & Bodansky, 1984; Steward & Young, 1984; Feinberg & Merrifield, 1975).

The 9-fluorenylmethoxycarbonyl (Fmoc) chemical method of peptide synthesis was developed by Carpino in 1972 and adapted to solid phase methods by Chang & Meienhofer (1978). The Fmoc protecting group replaced the tertiary butyloxycarbonyl (t-Boc) protecting group used by Merrifield solid phase chemistry. The use of the Fmoc protecting group allowed faster coupling times and milder reaction conditions using secondary amines such as piperidine, eliminating the repetitive trifluoroacetic acid treatments necessary in the t-Boc chemistry. A clear disadvantage of the Fmoc method at the present time is the lack of availability of Fmoc protected amino acids of the many other amino acids known to occur in nature outside the common twenty amino acids. However, in this project all the amino acids needed to synthesize the necessary peptides were available. For more details of this Fmoc method see Paivinen, et al. (1987). It should be noted that there are many manual and automated peptide synthesis methods using various chemistries each with its advantages and disadvantages (Kent, 1988). The t-Boc method, being the first developed, is the best understood and most common method of peptide synthesis.

The study of ligand-receptor binding has evolved many methods for determining various parameters of the biochemical and biophysical interaction of the ligand with the receptor molecule. The relationship between equilibrium binding constant K_D and allowable separation time is tabulated in Table 4-2. Included in separation time is the wash time. For the relatively weaker binding detection ability the separation time must be faster. For example, to detect binding and equilibrium constant of 10^{-7} M the separation time would need to be done in less than a second. Efforts have gone into developing different and improved methods of detecting weak binding. Table 4-3 lists some separation procedures from receptor-ligand studies along with associated separation time, advantages and disadvantages.

While doing receptor-ligand binding studies there are several potential artifacts that may occur. Table 4-4 lists examples of artifacts, the source of the problem and methods of detecting the artifact. These potential artifacts and their cause should be kept in mind and appropriate controls used to prevent erroneous conclusions.

Hill (1909) developed a mathematical formulation to describe the binding of several molecules that may have cooperative behavior as later applied to hemoglobin oxygen binding (Hill, 1910).

The hydrophilicity analysis of certain selected toxins used in this work helped to provide valuable clues into the chemical nature of the molecules. The GOR method also in some cases helped to picture the secondary structure of the selected toxins. But as with any predictive method they should only be used cautiously. The hydrophilicity analysis method was first published by Hopp and Woods in 1981 followed shortly by a similar method called hydropathy analysis published by Kyte and Doolittle in 1982. The Hopp and Woods method was based on the assigning of solvent parameters of each of the common twenty amino acids, as determined by Levitt in 1976, and summing the hexapeptide window. Hopp and Woods tested several window lengths and found that the six amino acid window optimized the prediction model for predicting the main antigenic sites. They found that the hexpeptide window method predicted all of the antigenic sites of the test protein molecules. They later published a HP basic computer program that could be adapted to other computers to serve as an analysis. Kyte and Doolittles' model works much the same except the window used was either a heptapeptide window or novapeptide window. On close examination of the parameters one sees that the

assigned values for the amino acids are opposite in sign than the Hopp and Woods. The tryptophan residue was given a hydrophilic value instead of the expected hydrophobic value. Since the introduction of these analysis programs much can be inferred about antigenicity, solvent solubility, and even protein-protein and protein-lipid interactions.

Secondary structure prediction methods have been used for many years with Chou and Fasman's method being most cited. The use of many tools, specifically, electron microscopy, Raman, CD/ORD, IR, NMR, X-ray have experimentally provide secondary structure information. Recently, Robson & Garnier (1986) published a computer model called the GOR method (Garnier, Osguthorpe and Robson) that predicts secondary stucture. The program assigns parameters that effect the folding, bond strength and flexibility, charge location and hydropathic index for a seventeen amino acid window and predicts four types of secondary structure for each amino acid of the primary sequence. The four types are the extended chain, random coil, helix, and turns. The statistical analysis of the program's predictive ability has scored it with an approximately 55% accuracy score which is slightly better than most other known prediction methods to-date and significantly better than the 25% chance value. The use of computer modeling should aid in the direct mapping and interaction of the neurotoxins with the receptors. Energy minimization calculations, x-ray crystallography coordinates and other spacial parameters can help model the likely conformation(s) of the bound ligand receptor (Garduno-Juarez et al., 1987; Gershoni, 1989; Shibata & Rein, 1989).

It is a well-established fact that snake venom postsynaptic neurotoxin binds to the acetylcholine receptor. Torpedo californica AChR is composed of five subunits, two of which are identical. Normally the receptor is expressed as $_2$. Acetylcholine is known to bind to the subunits of AChR. As two molecules of acetylcholine attach to two subunits, the AChR pore in the membrane opens up, allowing cations to pass through. This is the essence of nerve transmission from the nerve to the muscle. A snake postsynaptic neurotoxin is an antagonist to acetylcholine by competing with acetylcholine in attaching to the same site of AChR. When the neurotoxin attaches to the receptor, the AChR pore does not open and allow cations to pass through. This is the mechanism of neurotoxicity caused by postsynaptic neurotoxins.

An important question is: Which part(s) of the neurotoxin attaches to the AChR? In the past, the structure-function relationship of a neurotoxin was studied using a free neurotoxin (unbound to AChR). This type of study really does not tell which residues are involved in AChR binding. In order to solve this problem, we chemically modified the arginine residues of Lapemis neurotoxin under the condition of binding to AChR and then compared the result with that of unbound neurotoxin. One assumption is that the residues involved in the binding to AChR cannot be modified.

Lapemis toxin contains three arginine residues at positions 31, 34, and 37. In free (unbound) toxin Arg-31 and Arg-34 were modified, but Arg-37 was inaccessible to the modifying reagent presumably because it is embedded in the protein coiling. Figure 1-1 shows that Arg-31 and Arg-34 are relatively exposed to the outside. However, when arginine residues of the neurotoxin were modified using the Lapemis-AChR complex, neither Arg-31 nor Arg-34 were modified. The logical explanation is that both Arg-31 and Arg-34 are involved in AChR complexing.

Manual t-Boc synthesis presented problems in purification and identification of correct peptide; the syntheses appear to have many side reaction products, racemization and deletion peptides. t-Boc synthesis has several drawbacks. The repetitive acid (TFA) treatments can cause side reactions in amino acids and premature cleavage of the growing peptide chain from the support, and the final HF treatment frequently destroys peptide bonds (Paivianan et al.,1987).

Fmoc-synthesis appears to have overcome the synthesis technical problems, and the correct peptide products are the predominant peak of each HPLC chromatogram. The Fmoc peptides were made quickly by an automated synthesizer (Milligen 9050) and gave a high purity product that was confirmed by peptide sequencing. The purchase of the automated protein sequencer by this

Department has proven to facilitate sequence analysis. The use of the arylamine membrane and manufacture protocol to covalently link the carboxyl groups of the peptides serves well. The coupling efficiency however, was only 24% initial yield but due to the ample amount of peptide samples available this presented no real problem. The low yield may have been due to improper storage of the membrane disks upon arrival which were left at room temperature instead of being refrigerated as stated by the manufacture. The computerized Edman sequencing was monitored at 269 nm and 313 nm and the results of the previous cycle was dynamically subtracted to aid in the correct assignment of the current cycle amino acid.

Since peptide B_1 differs from B_2 in the fact that the B_1 peptide contains the disulfide bond due to the terminal cysteine residues while peptide B_2 does not contain the cysteine residues and therefore does not contain the disulfide bond suggests the importance of the disulfide bond. Future work will be several related binding study experiments to further clarify the importance of the disulfide bond. The B_1 peptide will be carboxymethylated forcing an open conformation and radiolabelled. The binding study with CM-peptide should confirm or disprove the importance of the disulfide bond.

The results of this study will determine which region(s) of the neurotoxin that are important in receptor binding and will allow the designing of future experiments aimed at elucidating which amino acids of the neurotoxin are directly involved in binding with the acetylcholine receptor. It appears the peptides may be relatively non-toxic which may well be a plus since antibodies raised against these peptides may recognize and neutralize the Lapemis toxin in-vivo and perhaps other related neurotoxins. It should be noted that our hydrophilicity analysis of Lapemis toxin suggests that the loop B should be the prime candidate for serving as the non-toxic antigen based on its high hydrophilicity and correlation of high hydrophilicity to antibody production (method and correlation, Hopp and Woods, 1981, 1983).

Fractionation patterns for 1989 and 1986 Lapemis venom samples were virtually identical, indicating that the extraction procedures had yielded the same product from both batches of venom glands. The 1986 crude venom has been used for isolating Lapemis neurotoxin for subsequent modification and binding studies (e.g. Lin and Tu, 1988), and it appears that the 1989 sample will yield neurotoxin as well.

The Fmoc peptides were made very quickly by an automated synthesizer and gave a high purity product. The use of a nonsense peptide was also made to serve as a negative control to eliminate any artifacts that may be introduced by the method of synthesis and preparation.

The results of this study will determine which region(s) of the neurotoxin that are important in receptor binding and will allow the designing of future experiments aimed at elucidating which amino acids of the neurotoxin are directly involved in binding with the acetylcholine receptor. It appears the peptides may be relatively non-toxic which may well be a plus since antibodies raised against these peptides may recognize and neutralize the Lapemis toxin in-vivo.

Acetylcholine Receptor Binding Assay.

Binding of the synthetic peptides to the nicotinic acetylcholine receptor will be determined by their ability to compete with Lapemis ^{125}I-neurotoxin.

The attempt to measure the binding of the synthetic peptides will be by determining the decrease in ^{125}I-neurotoxin binding to the receptor following preincubation of the receptor with varying amounts of synthetic peptides for at a selected time, perhaps 1 hr in a competition assay. The dissociation (binding) constant of Fmoc-synthesis appears to have overcome the synthesis technical problems, and the correct peptide products are the predominant peak of each HPLC chromatogram. The Fmoc peptides were made very quickly by an automated synthesizer and gave a high purity product. The use of a nonsense peptide was also made to serve as a negative control to eliminate any artifacts that may be introduced by the method of synthesis and preparation.

The relative binding constants of each Fmoc synthetic peptide are to be determined using the established binding assay (Schmidt and Raferty, 1979) and Scatchard analysis (Scatchard, 1949), followed by the relative toxicity determinations.

The results of this study will determine which region(s) of the neurotoxin that are important in receptor binding and will allow the designing of future experiments aimed at elucidating which amino acids of the neurotoxin are directly involved in binding with the acetylcholine receptor. It appears the peptides may be relatively non-toxic which may well be a plus since antibodies raised against these peptides may recognize and neutralize the Lapemis toxin in-vivo.

Ruan et al. (1990) have shown the systematic study of the long chain post-synaptic neurotoxin synthetic fragments with those of the AChR. Boyot et al. (1990) have shown that the use of another important strategy (recombinant technology) will add greatly to the goals of this research.

Both Arg-31 and Arg-34 of Lapemis toxin are involved in AChR complexing. Peptide B_1 binds the AChR while peptide B_2 appears not to bind at all. Since peptide B_1 differs from B_2 in the fact that the B_1 peptide contains the disulfide bond (due to the terminal cysteine residues) while, peptide B_2 does not contain the cysteine residues and therefore does not contain the disulfide bond suggests the importance of the disulfide bond in a probable structural role.
With comparisons this work and that of others, the critical amino acids and regions of the toxin and the AChR which are involved in binding and structure-function are becoming increasing more clear.

II. Isolation and Search for Lapemis Toxin Precursor

The molecular weight of the acidic 13.5 kD protein is somewhat high for a potential neurotoxin precursor, but it is in the correct range for the common venom enzyme phospholipase A2. Subsequent assays confirmed that this protein is indeed a phospholipase A2.

Seven more tryptic peptides of Lapemis parvalbumin will be sequenced. To obtain overlapping fragments, the reduced and alkylated protein will be digested with Glu-C endopeptidase. The possible role of parvalbumin in potentiating toxicity of PLA2 will be investigated. Further sequencing efforts are also being directed toward the PLA_2 from Lapemis venom.

Biological activity of Gila toxin, isolated from Heroderma horridum horridum causes the hind limb paralysis, pain, and loss of equilibrium (gyration) in mice. However Gila toxin did not cause any hemorrhage in skin or systemic hemorrhage such as exophthamial. Gila toxin is a serine type protease. And a thrombin like enzyme which cleaves A , B , and chain at a very slow rate which differs from snake thrombin like enzyme such as Crotalus atrox which cleaves only A , and B at a very fast rate (within 1 hr) but does not cleave the chain. The cleavage sites on fibrinogen between Gila toxin and C. atrox are also different as indicated by SDS-PAGE.

Gila toxin is also a kallikrien-like enzyme which cleaves HMW kininogen and releases bradykinin. Bradykinin is 9 amino acids long and known to be a very potent agent that causes hypotension and pain. It is probable that injection of Gila toxin in mice releases bradykinin from kininogen, therefore results in pain. Hind limb paralysis and loss of equilibrium caused by injection of Gila toxin may also be caused by rising blood bradykinin. Since bradykinin is a hypotensive agent, increase of this agent causes low blood pressure which may result in hind limb paralysis and loss of equilibrium. Hind limb paralysis was also observed over 24 hr after Gila toxin injection.

Lower blood pressure in the body could be compensated for by activating the angiotensin pathway. Angiotensin is a hypertensive agent which can increase blood pressure. Therefore, the body should recover soon after injection with Gila toxin. However, figure 4-4 shows that Gila toxin also cleaves angiotensin I at the first two amino acid residues. Removing the first two amino acid residues from both angiontensin I and II results in a drop of angiotensin activity from 100 to 0.3% (Jorgensen et al., 1970). Therefore the body is unable to compensate for low blood pressure by angiotensin pathway system when injected with Gila toxin.

Section 5 Figures

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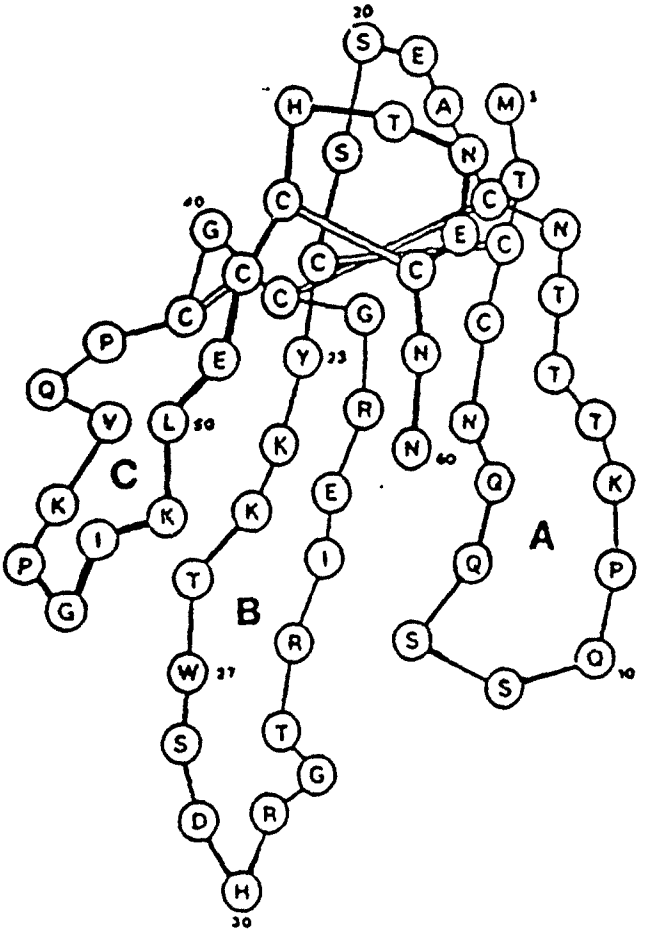


Figure 1-1 The Primary Sequence and Tertiary Structure of Lapemis Toxin.

The tertiary structure of Lapemis toxin is based on the crystal structure of a similar sea snake neurotoxin, toxin b found in *Laticauda semifaciata* (Tsernoglou and Petsko, 1976). The letters inside the circles are the single letter designation of the amino acids.

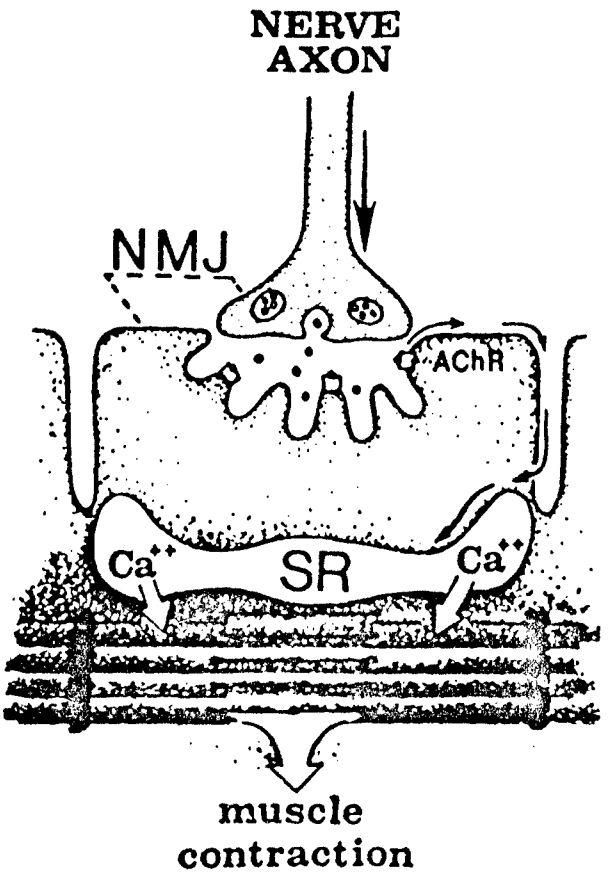


Figure 1-2 Depiction of the Neuromuscular Junction
During neurotransmission the action potential passes down the motor neuron axon resulting in release of acetylcholine into the neuromuscular junction. The acetylcholine then binds the acetylcholine receptor imbedded in the muscle cell membrane. The ligand gated receptor undergoes a conformational change that allows cations (usually sodium ions) to enter the cell producing a depolarization of the membrane potential. (Courtesy of Dr. A. T. Tu)

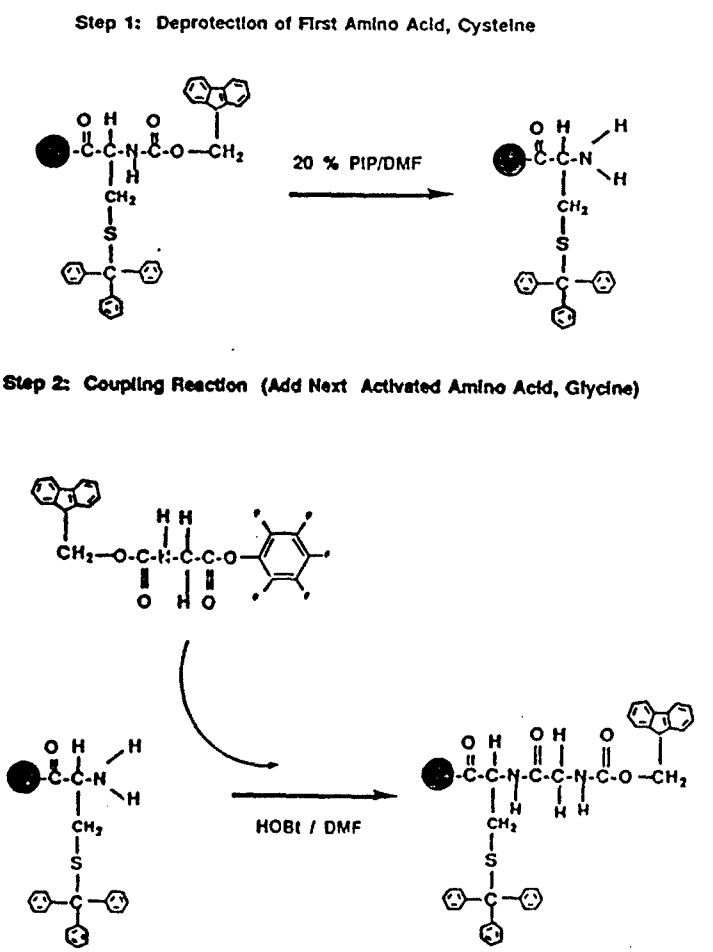


Figure 2-1 Summary of the Fmoc Chemistry Cycle for Peptide Synthesis

The figure depicts one cycle of the automated Fmoc Chemistry used to make the peptides. See text for details.

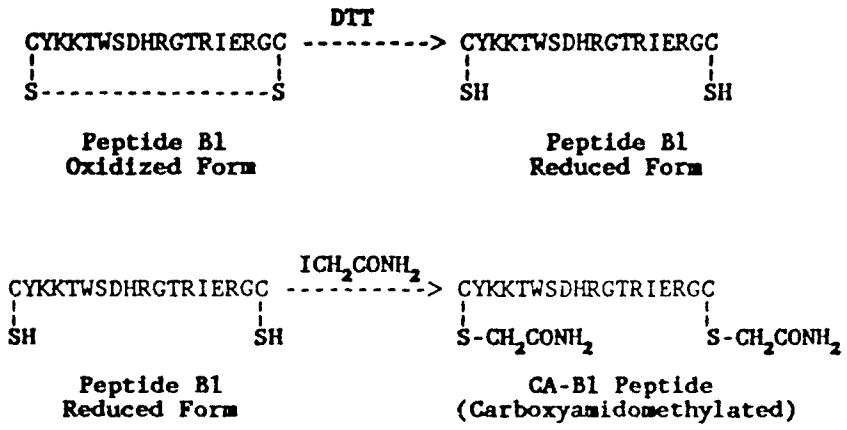


Figure 2-2 CA-Modification Chemistry

The disulfide bonds of the peptide B1 were first reduced using dithiothreitol producing the free sulfhydryls (SH). The reduced form was then alkylated using iodoacetamide producing the carboxyamidomethylated peptide B1.

All procedures are done at 4°C

**Mince electroplax tissue (75-100 g)
and place in 200 ml of buffer A
in a blender 8 X 15 sec @ high setting
and strain through wire mesh or cheese cloth**

↓

**Centrifuge 10 min @ 2,000 x g
and collect supernatant**

↓

**Centrifuge 60 min @ 25,000 x g
and resuspend pellet and homogenize
(Dounce homogenizer) in buffer B**

↓

**Mix with Virtis mixer for 30 min
and centrifuge 30 min @ 65,000 x g
collect supernatant**

↓

**Combine supernatant with cobrotoxin
linked Sepharose 4B resin and mix gently 1 h**

↓

**Pour affinity column with the above resin
and wash with 300 column volumes buffer C**

↓

**Transfer resin back to beaker with 25 ml
buffer D and mix for 12 to 15 h**

↓

**Repour Affinity column and collect elutant
which contains the purified AChR and dialyze
first against 500 volumes of buffer E for 24 h
followed by 500 volumes of buffer F for 24 h**

↓

Collect purified AChR dialysate and store -70°C

Figure 2-3 AChR Isolation Protocol

The above isolation protocol was used to purify the solubilized acetylcholine receptor. See the text for the buffers.

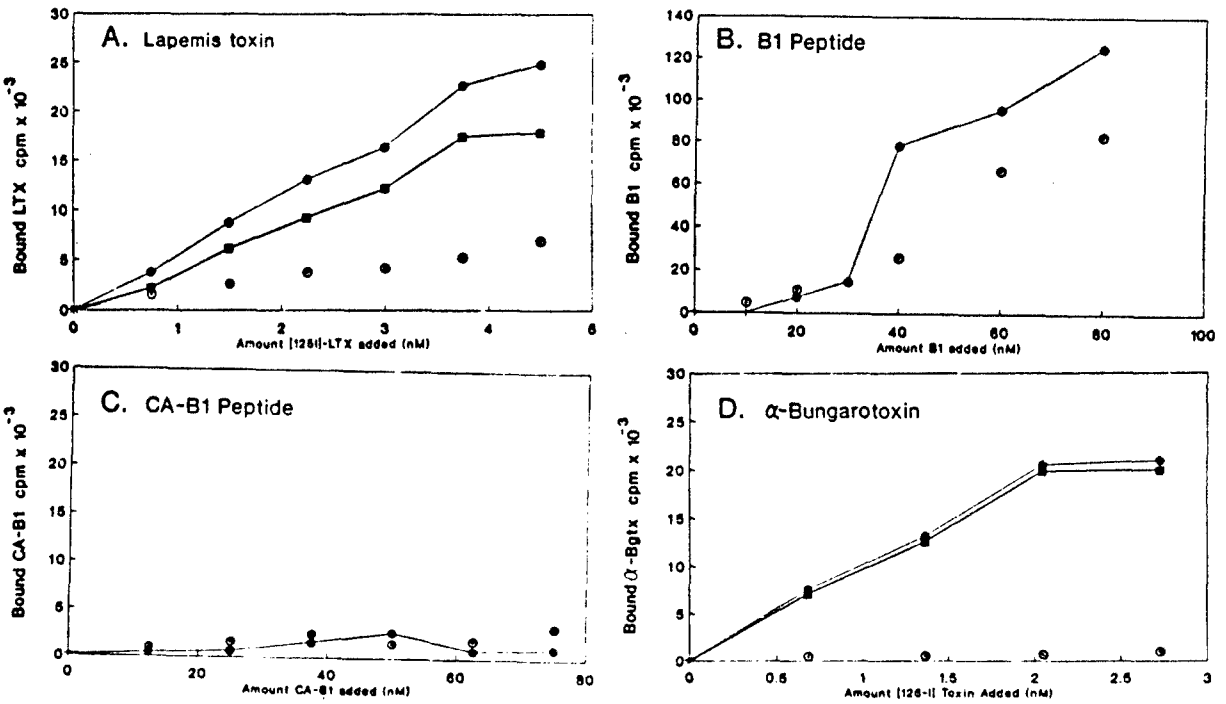


Figure 3-1 Binding Study Plots

(A) Lapemis toxin; (B) Peptide B1; (C) CA-B1 Peptide and (D) α-Bungarotoxin. Solid circles are total binding; open circles indicate the non-specific binding; while the solid squares indicate the specific binding.

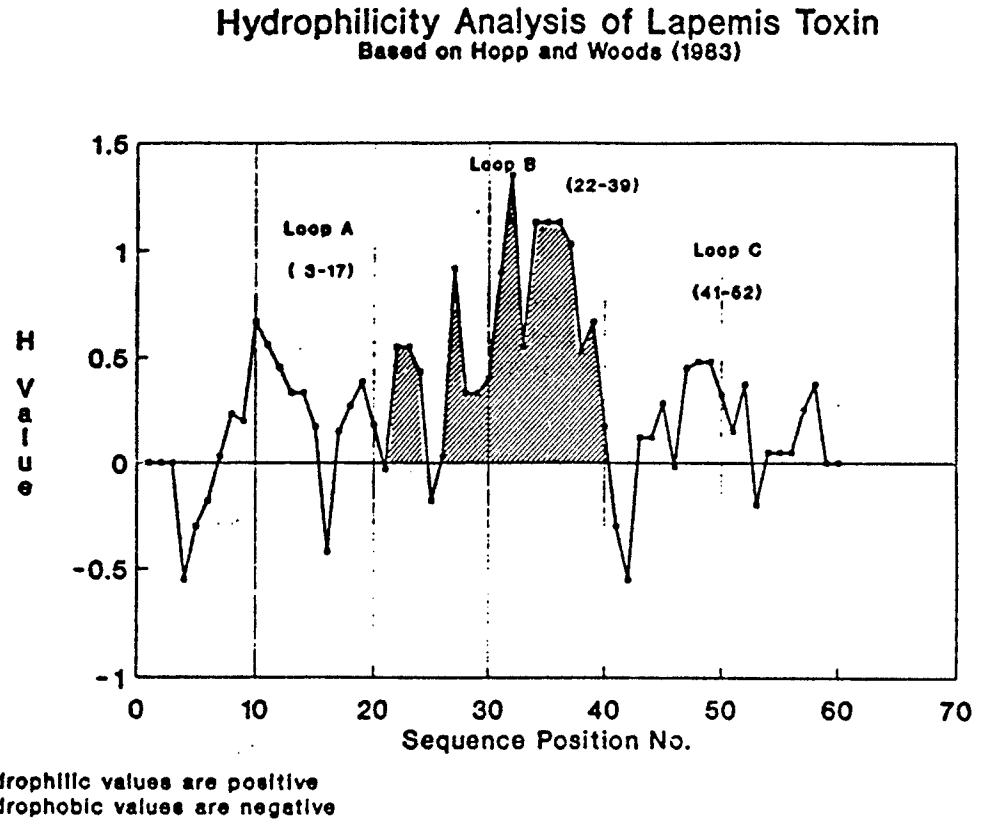


Figure 3-2 Hydrophilicity Analysis of Lapemis Toxin

Using the sknown sequence of LTX, the method of Hopp and Woods (1983) was applied. The method assigns average hydrophilicity values to sequence position numbers based on a moving hexapeptide window.

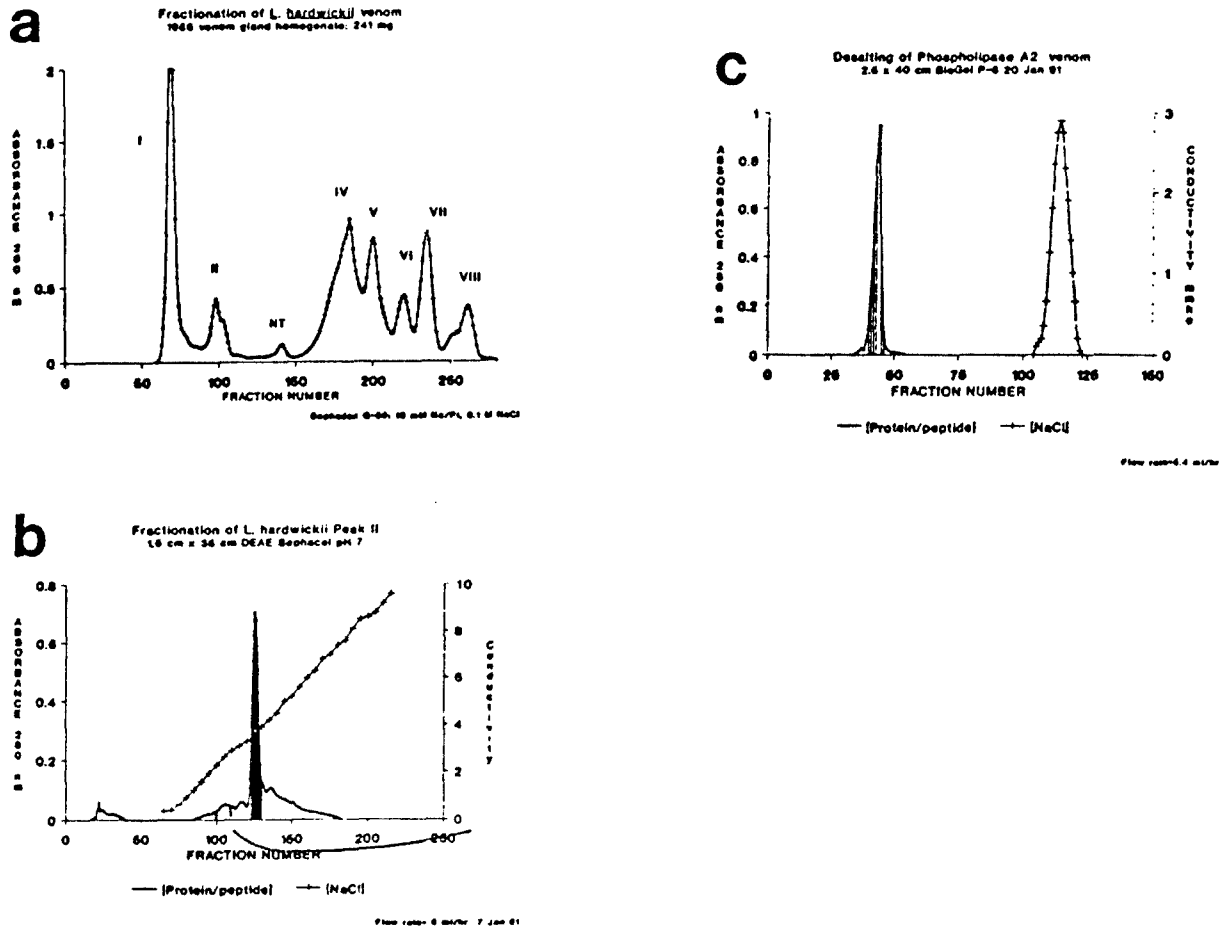


Figure 3-3. Fractionation of Lapemis venom.
The phospholipase A_2 from L. hardwickii venom is an acidic protein, and its isoelectric point is approximately 4.6. Molecular weight estimates indicate that it is a small protein of 13.5-14 kD. The enzyme hydrolyzes of phosphatidylcholines, and it shows much greater activity toward dipalmitoyl phosphatidylcholine (62.7 μmol fatty acid released/mg protein/minute) than toward diarachidoyl phosphatidylcholine (in the assay system used).

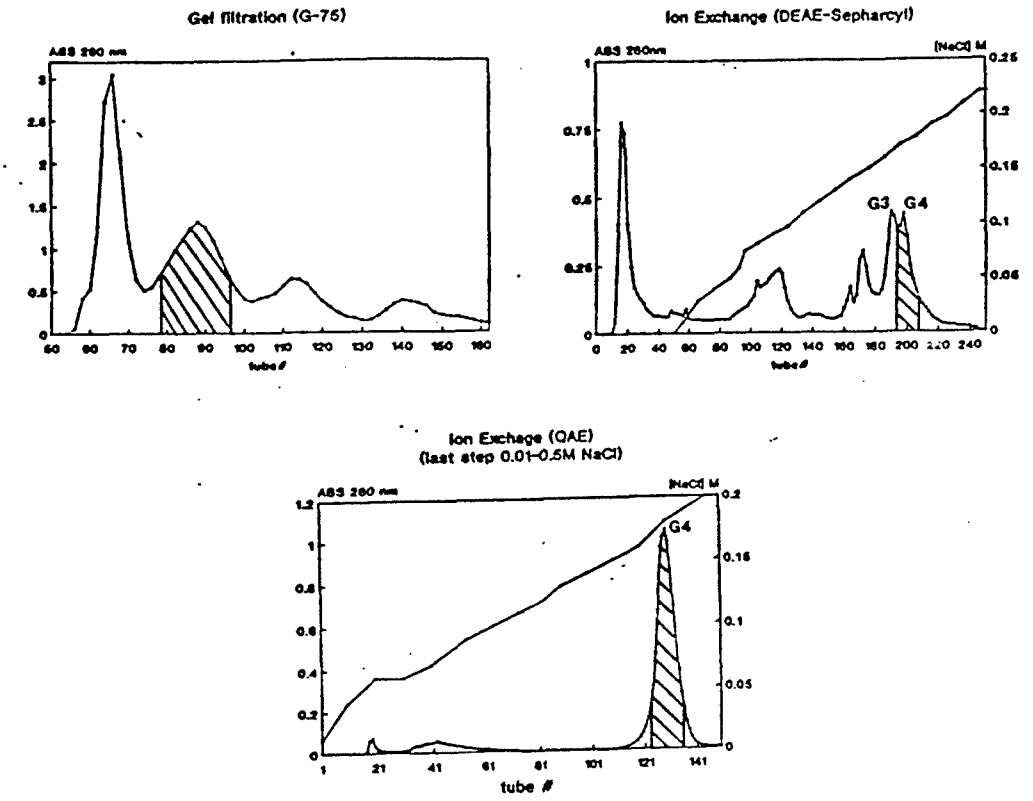
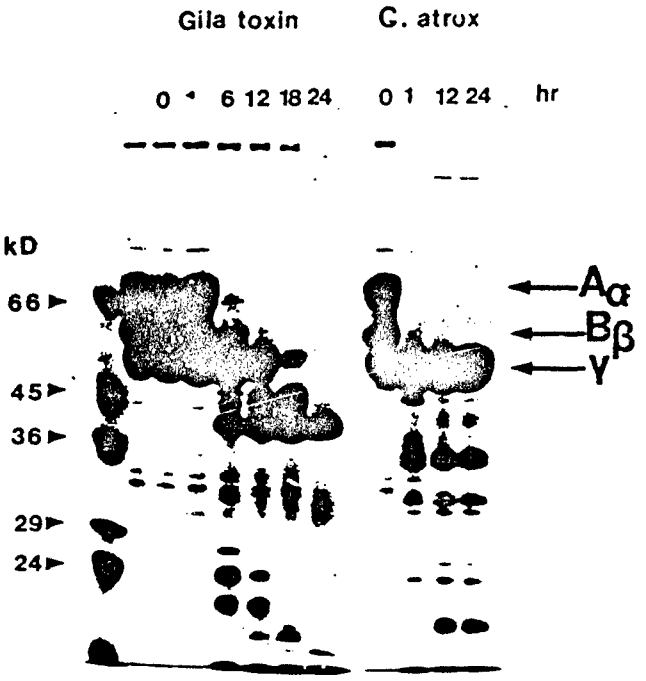


Figure 3-4. Isolation of Gila toxin

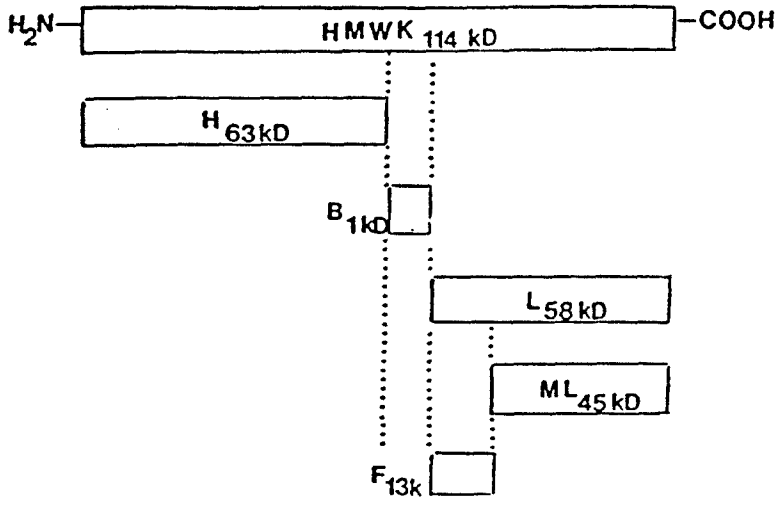
Column chromatography of Gila toxin isolation. Crude H. horridum was dissolved in 0.05 M Tris-HCl, pH 8.5 containing 0.1 M NaCl and was loaded onto Sephadex G-75 column. The protein was eluted with the same buffer at a flow rate of 10 ml/hr. The second peak was pooled and dialyzed, then loaded onto DEAE-ion exchange column equilibrated with 0.05 M Tris pH 7.5 and 0.01 M NaCl. The column was eluted with linear NaCl gradient at flow rate of 12 ml/hr. The G4 peak was pooled and dialyzed, then loaded onto a QAE ion exchange column which was equilibrated with the same buffer as the previous step. The column was eluted with a linear NaCl gradient at a flow rate of 12 ml/hr. The G4 peak was pooled, dialyzed and lyophilized.



SDS-PAGE analysis of reduced human fibrinogen after digestion by Gila toxin and Crotalus atrox venom. Lanes 3-8 depict 2% fibrinogen samples after incubation with 50 µg of Gila toxin for the specificed times. Lanes 10-13 depict 2% fibrinogen samples after incubation with 50 µg crude C.atrox for the specificed times.

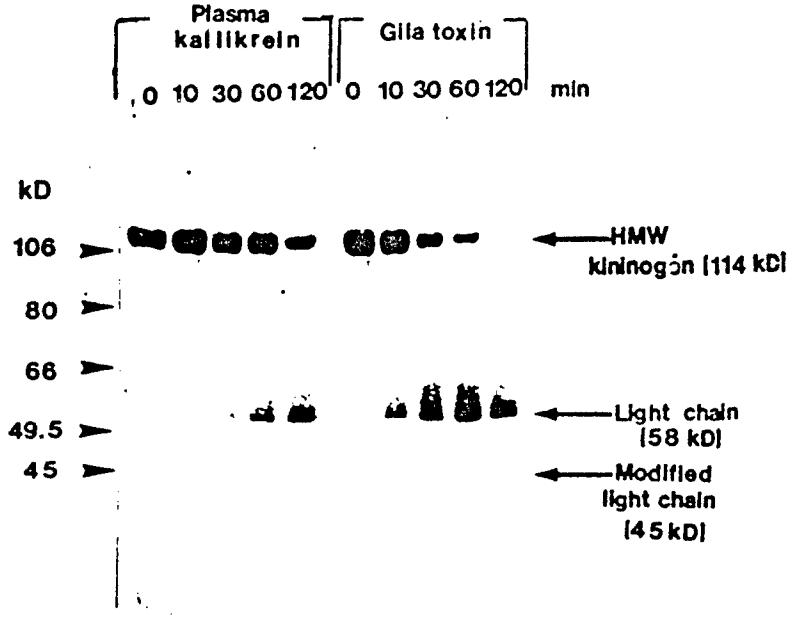
Figure 3-5 Fibrinogenolytic Activity of Gila toxin

Proteolytic digestion of HMW kininogen with plasma kallikrien



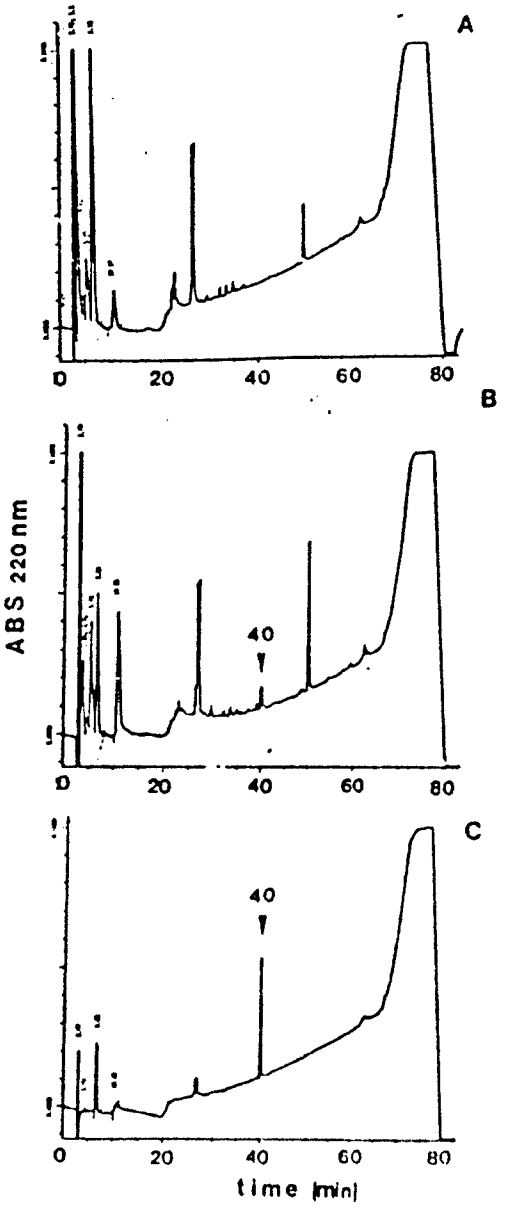
HMWK = High Molecular Weight Kininogen
H = Heavy Chain
L = Light Chain
ML = Modified Light Chain
B = Bradykinin
F = Fragment

Figure 3-6 Degradation of HMW Kininogen



SDS-PAGE analysis of degraded of High Molecular Weight (HMW) kininogen after digested by plasma kallikrien or Gila toxin. Lanes 1-5 show HMW kininogen after incubation with plasma kallikrien for specificed times. Lanes 7-11 show HMW kininogen after incubation with Gila toxin for specificed times.

Figure 3-7 SDS-PAGE of Degradation of HMW Kininogen



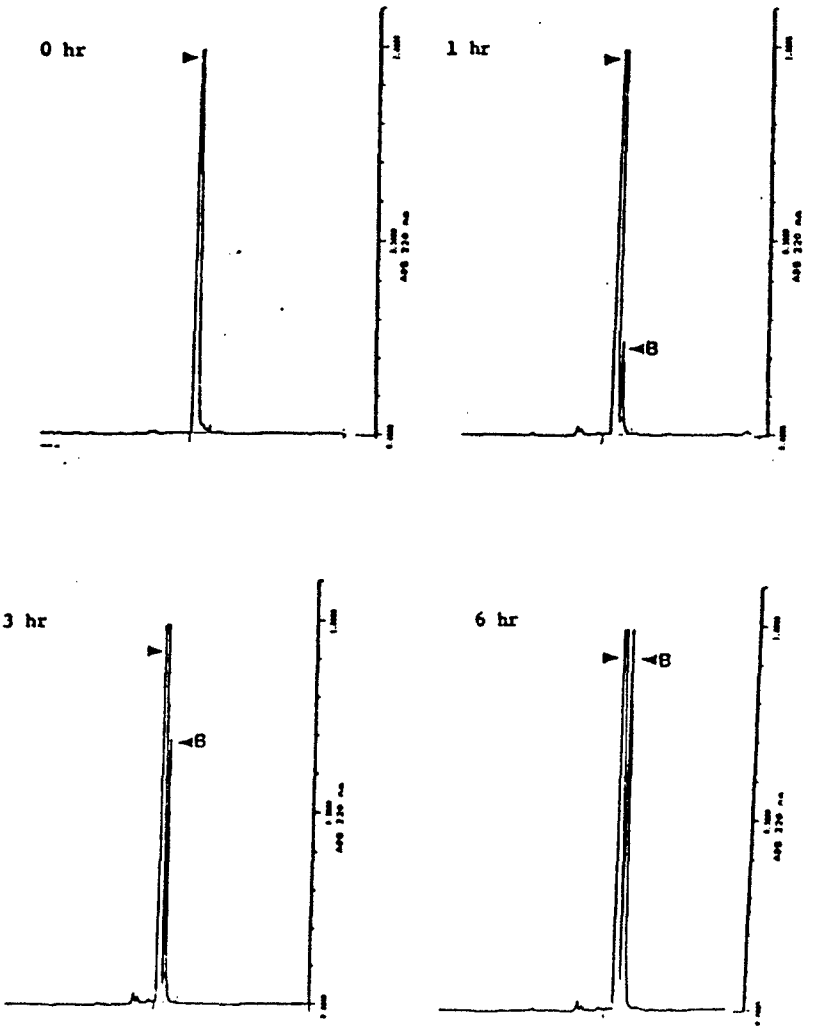
HPLC chromatograph of bradykinin release by Gila toxin. High Molecular Weight kininogen was incubated in the absence (A) or presence (B) of Gila toxin as described under Methods. (C) is HPLC chromatograph of bradykinin. Chromatography was performed by using a linear gradient from 0-50% acetonitrile in water, containing 0.1% TFA and a flow rate of 1 ml/min.

Figure 3-8 Bradykinin Release HPLC Isolation

Bradykinin	R P P G F S P F R
B_{40}	R P P G F

Comparision of N-terminal amino acid sequences of B_{40} peptide and bradykinkin amino acid sequences.

Figure 3-9 Sequence of Released Peptide (BK)



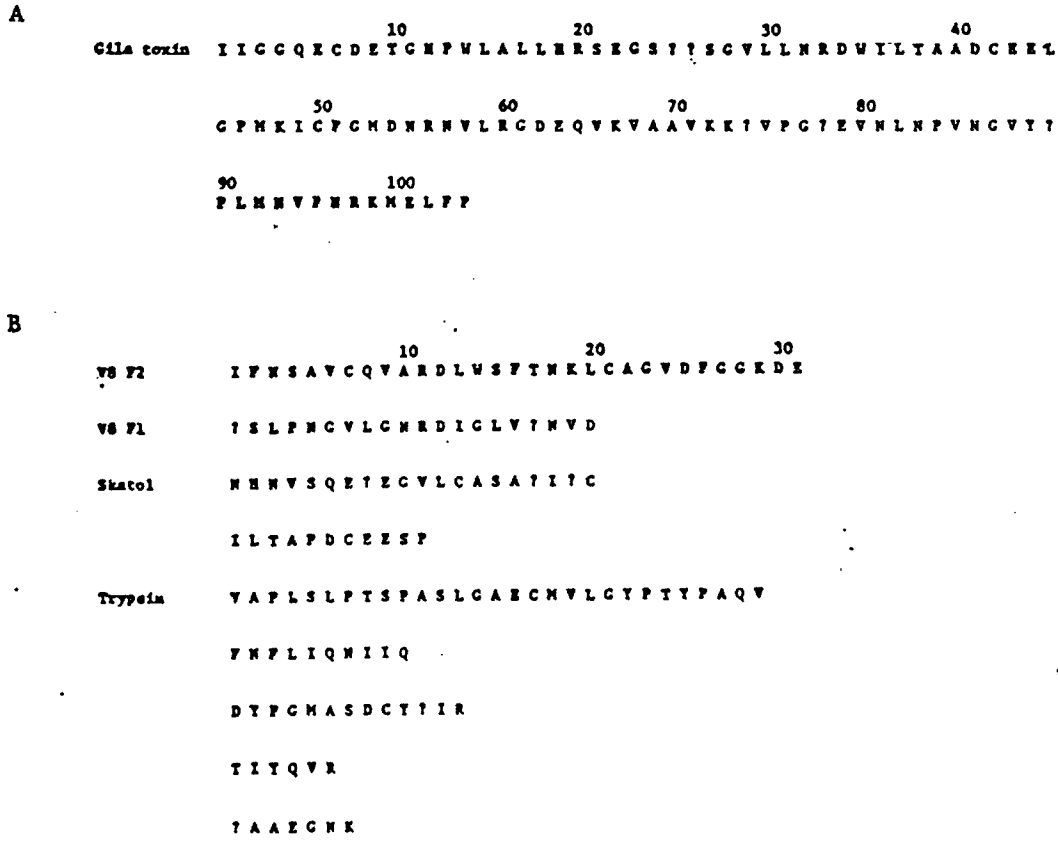
HPLC chromatographs of the results of angiotensin I cleavage by Gila toxin. Each chromatograph represents angiotensin I and degradation product after specificed incubation times. Chromatography was performed by using a linear gradient from 0-50% acetonitrile in water, containing 0.1% TFA, for duration of 50 min and a flow rate of 1 ml/min.

Figure 3-10 Digestion of Angiotensin I

peptide B	V Y I H P F H L
Angiotensin I	D R V Y I H P F H L
Angiotensin II	D R V Y I H P F
Angiotensin III	R V Y I H P F

Comparision of amino acid sequeces B peptide, angiotensin I, II, III.

Figure 3-11 Peptide Comparison to Angiotensin I, II and III



Partial amino acid sequences of Gila toxin. (A) the Gila toxin N-terminal sequences. (B) sequences from cleavage fragments by V8, skatol, trypsin.

Figure 3-12 Gila Toxin Peptide Sequences

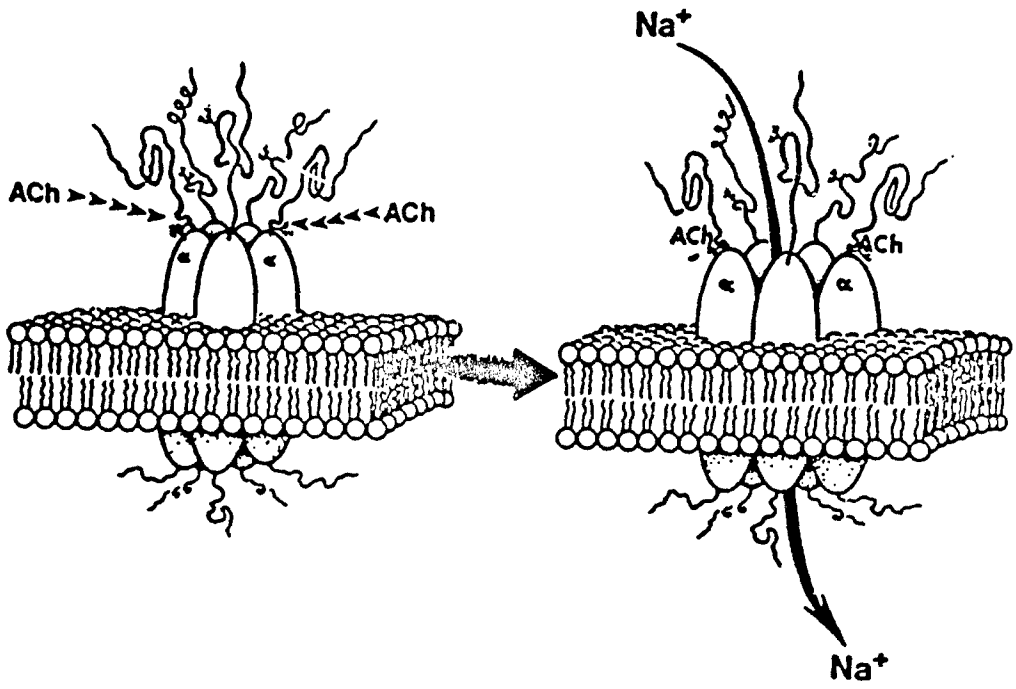


Figure 4-1 Diagram of the Membrane Acetylcholine Receptor Interacting with Acetylcholine and a Post-Synaptic Neurotoxin such as Lapemis Toxin

The acetylcholine neurotransmitter (ACh) released from the motor axon binds to the α-subunits of the acetylcholine receptor allowing the receptor to open allowing cations, especially sodium ions to enter and depolarize the muscle cell membrane. Post-synaptic neurotoxins block the binding of acetylcholine and the receptor opening.

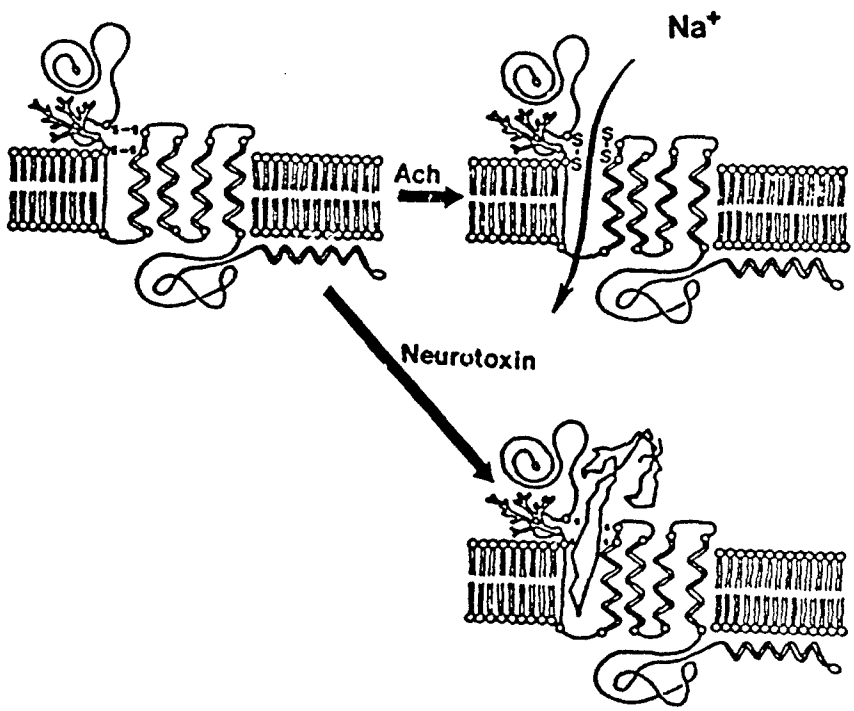
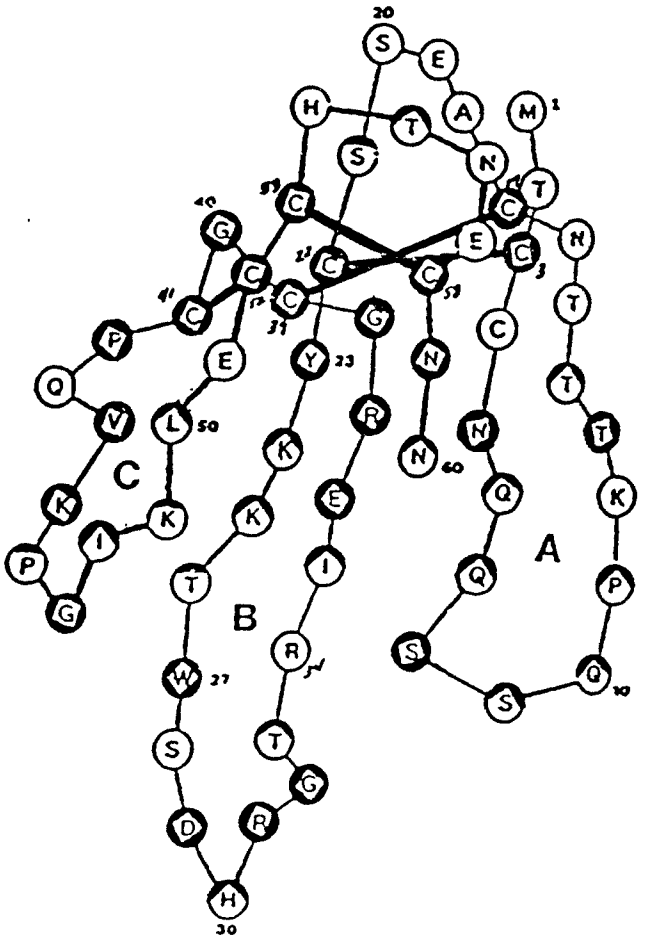


Figure 4-2 Alpha subunit AChR

The figure depicts the binding of the acetylcholine (ACh) which allows the conformational change of the ligand gated receptor cation channel. Upon binding the receptor opens allowing predominately sodium ions to enter the muscle cell depolarizing the membrane. The strong neurotoxin binding competes with acetylcholine binding and blocks the conformational opening of the receptor.



- Invariant
- > 66% conserved
- 33%-66% conserved
- < 33% conserved

Figure 4-3 Conserved Amino Acids of Lapemis Toxin

The sequences of neurotoxins from Elapidae and Hydrophiidae venoms have been compared and percent of conservation determined. From this figure certain invariant amino acids are readily seen. Also, non-conserved residues are noted. (Adapted from Dufton and Hider, 1983)

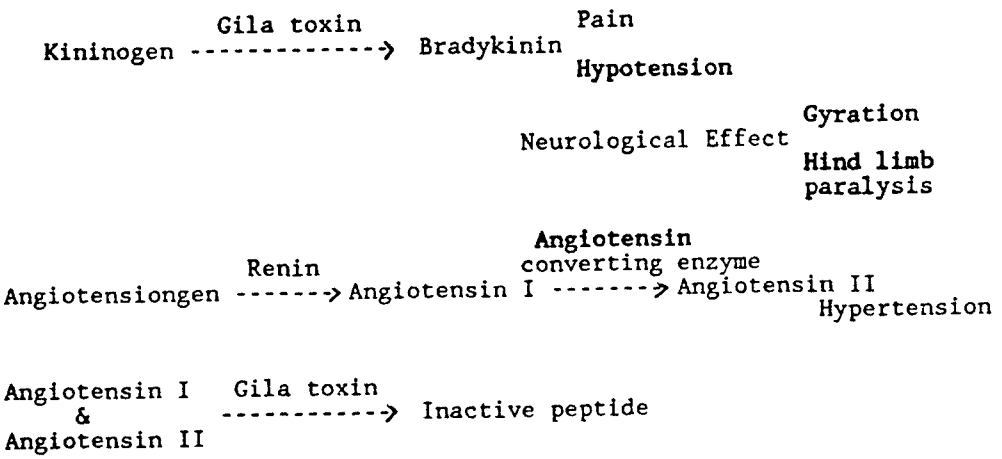


Figure 4-4 Gila Toxin Actions

Section 6 Tables

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Table 1-1. Classification of Sea Snake Family Hydrophiidae (Smith, 1926)

Subfamily	Genus	Species
Laticaudinae	*Laticauda*	*laticaudata*
		colubrina
		crockeri
		semifasciata
		schistorhynchus
	Aipysurus	***eydouxi***
		fuscus
		tenuis
		laevis
		duboisii
		foliosquama
		apraefrontalis
	Emydocephalus	***annulatus***
		ijimae
Hydrophiinae	*Hydrelaps*	*darwiniensis*
	Kerilia	***jerdoni***
	Thalassophina	*viperina*
	Enhydrina	***schistosa***
	Hydrophis	***nigrocinctus***
		kingi
		mertoni
		spiralis
		melanosoma
		belcheri
		elegans
		cyanocinctus
		semperi
		malanocephalus
		obscurus
		klossi
		major
		bituberculatus
		stricticollis
		torquatus
		ornatus
		inornatus
		lapemoides
		mamillaris
		caerulescens
		fasciatus
		brookii
	Acalytophis	*peronni*
	Thalassophis	*anomalous*
	Kolpophis	*annandalei*
	Lapemis	*hardwickii*
		curtis
	Astrotia	*strokesii*
	Pelamis	*platurus*
	Microcephalophis	*gracilis*
		cantoris

Table 1-2. Snake Venom Protein Components Found in Sea Snake Venoms

Components	Comment	References
Acetylcholinesterase	activity detected	Tu and Toom, 1971; Gawade and Bhide, 1977; Su et al., 1984
Hyaluronidase	activity detected	Tu and Toom, 1971
Leucine aminopeptidase	activity detected	Tu and Toom, 1971
5'-Nucleotidase	activity detected	Gawade and Bhide, 1977; Su et al., 1984; Setoguchi et al., 1968
Phosphodiesterase	activity detected	Tu and Toom, 1971; Setoguchi et al., 1968; Su et al., 1984
Phosphomonoesterase	isolated	Su et al., 1984; Uwatoko-Setoguchi, 1970; Setoguchi et al., 1968
Phospholipase A_2	several sequenced	Carey and Wright, 1960; Ibrahim and Thompson, 1965; Tu and Toom, 1971; Fohlman and Eaker, 1977; Tan, 1982; Su et al., 1984; Durkin et al., 1981; Uwatoko-Setoguchi et al., 1968; Tu et al., 1970; Yoshida et al., 1979; Nishida et al., 1982; Durkin et al., 1981
Postsynaptic α-Neurotoxin	31+ sequenced	See Table 1-5

Table 1-3. Known Snake Venom Components Not Detected or Not Studied in Sea Snake Venoms

Component	References
Acetylcholinesterase Inhibitors	
Adenosine Triphosphatase	
L-amino acid oxidase	**Gawade and Bhide, 1977; Su et al., 1984**
Amylase	
Angiotensin Converting Enzyme Inhibitors	
Arginine esterases	Gawade and Bhide, 1977; Su et al., 1984
β-Glucosamidase	
Bradykinin Potentiator Peptides	
Cardiotoxins	
Cardiotoxin Like Basic Peptides	
Capillary Permeability Factor(s)	
Catalase	
Cobra Venom Factor	
Deoxyribonuclease	
Direct Lytic Factor(s)	
Endopeptidases	
Endonucleases	
Factor X activator	
Factor V activator	
Fibrinogenolytic Factors	
Fibrinolytic Factors	
Glutamic-Pyruvic Transaminase	
Glycerophosphatase	
Hemolytic Factor(s)	
Hemorrhagins	
Heparinase Like Enzyme	
Kininogenase	
Lactate Dehydrogenase	
Membranotoxins	
Myotoxins	
Nerve Growth Factor	
NAD-nucleosidase	
Phospholipase B	
Potassium Channel Blockers	
Postsynaptic -Neurotoxins	
Presynaptic β-Neurotoxins	
Proteases	Uwatoko et al., 1966a
Prothrombin activator	
Proteinase inhibitors	
Ribonuclease	
Thrombin-Like Enzymes	

References (Tu, 1991; Tu, 1988; Shier & Mebs, 1990;Lee, 1979; Tu, 1977; Rosenberg, 1978)

Table 1-4. Toxicities of Sea Snake Venoms and Components*

Venom or Component	LD50 (mg/Kg)	Route /Animal	References
Acalyptophis peronii			
Venom	0.079	sc Mouse	Minton, 1983
Major Toxin	0.125	iv Mouse	Mori & Tu, 1988a
Minor Toxin	0.10	iv Mouse	Mori & Tu, 1988b
Aipysurus duboisii			
Venom	0.044	sc Mouse	Minton, 1983
Aipysurus eydouxii			
Venom	>4.00	iv Mouse	Tu, 1974
Aipysurus laevis			
Venom	0.05	im Mouse	Barber et al., 1974
	0.13	im Mouse	Tamiya & Puffer, 1974
	0.26	sc Mouse	Baxter & Gallichio, 1976
	0.084	sc Mouse	Minton, 1983
	0.45	sc Mouse	Minton, 1983
Laevis toxin	0.076	im Mouse	Maeda & Tamiya, 1978
Astrotia stokesii			
Toxin a	0.13	im Mouse	Maeda & Tamiya, 1978
Toxin b	0.096	im Mouse	Maeda & Tamiya, 1978
Toxin c	0.098	im Mouse	Maeda & Tamiya, 1978
Emydocephalus annulatus			
Venom	>25.00	sc Mouse	Minton, 1983
Enhydrina schistosa			
Venom	3.50(mg/70kg)	Man	Rogers, 1902-1903
	0.107	ip Mouse	Carey & Wright, 1960
	0.125	iv Mouse	Barme, 1963, 1968
	2.10-2.50(μg/animal)	Mouse	Barme, 1963, 1968
	0.35	iv Mouse	Cheymol et al., 1967
	0.150	sc Mouse	Minton, 1976
	90.00	iv Mouse	Tu and Ganthavorn, 1969
	0.01-0.09	iv Mouse	Chang, 1979
	0.05-0.20	sc Mouse	Chang, 1979

Table 1-4. Toxicities of Sea Snake Venoms and Components (Continued)

Venom or Component	LD50 (mg/Kg)	Route /Animal	References
Enhydrina schistosa			
Venom	0.03	iv Rabbit	Chang, 1979
	3.60(mg/70kg)	Man	Reid, 1979
Year of Collection (1967)	0.14	iv Mouse	Tu, 1974
(1969)	0.21	iv Mouse	Tu, 1974
Major toxin	0.04	iv Mouse	Tu & Toom, 1971
Enhydrotoxin a	0.042	iv Mouse	Gawade & Gaitonde, 1982a
Enhydrotoxin b	0.045	iv Mouse	Gawade & Gaitonde, 1982b
Enhydrotoxin c	0.052	iv Mouse	Gawade & Gaitonde, 1982b
CM-IV-Sa	0.07	im Mouse	Gawade & Bhide., 1978
Hydrophis belcheri			
Venom	0.07	im Mouse	Barber et al., 1974
	0.24	im Mouse	Tamiya & Puffer, 1974
Hydrophis cyanocintus			
Venom	0.24	ip Mouse	Carey & Wright, 1960
	4.80(μg/animal)	Mouse	Barme, 1963, 1968
	0.35	iv Mouse	Barme, 1963, 1968
	0.67	iv Mouse	Cheymol et al., 1967
	0.35	iv Mouse	Tu & Ganthavorn, 1969
	0.464	sc Mouse	Baxter & Gallichio, 1976
Toxin	0.05	iv Mouse	Su et al., 1984
Hydrophis elegans			
Venom	0.12	im Mouse	Barme, 1963
	0.03	im Mouse	Barber et al., 1974
	0.26	sc Mouse	Baxter & Gallichio, 1976
Hydrophis fasciatus			
Venom	0.175	iv Mouse	Barme, 1963
Hydrophis klossi			
Venom	0.20-0.53	ip Mouse	Carey & Wright, 1960
	4.0-10.60(μg/animal)	Mouse	Barme, 1963

Table 1-4. Toxicities of Sea Snake Venoms and Components (Continued)

Venom or Component	LD50 (mg/Kg)	Route/Animal	References
Hydrophis major			
Venom	0.193	sc Mouse	Baxter & Gallichio, 1976
Hydrophis melanocephala			
Venom	0.082	im Mouse	Tamiya & Puffer, 1974
	0.111	sc Mouse	Minton, 1983
Hydrophis melanosoma			
Venom	0.40	ip Mouse	Carey & Wright, 1960
	8.00(μg/animal)	Mouse	Barme, 1963
Hydrophis nigrocinctus			
Venom	0.343	sc Mouse	Baxter & Gallichio, 1976
Hydrophis ornatus			
Venom	2.2	iv Mouse	Tu, 1974
	0.12	im Mouse	Baxter & Gallichio, 1976
Hydrophis spiralis			
Venom	0.25-0.38	ip Mouse	Carey & Wright, 1960
	5.00(μg/animal)	Mouse	Barme, 1963
Hydrophis stricticollis			
Venom	0.164	sc Mouse	Baxter & Gallichio, 1976
Kerilia jerdoni			
Venom	0.53	ip Mouse	Carey & Wright, 1960
	10.60(μg/animal)	Mouse	Barme, 1963, 1968
Lapemis hardwickii			
Venom	0.26	ip Mouse	Carey & Wright, 1960
	0.20	iv Mouse	Barme, 1963
	5.20-5.70(μg/animal)	Mouse	Barme, 1963
	0.44	iv Mouse	Cheymol et al., 1967
	0.71	iv Mouse	Tu & Ganthavorn, 1969
	0.70	iv Mouse	Tu & Heng, 1971
	0.16	im Mouse	Tamiya & Puffer, 1974
Year of collection (1967)	0.71	iv Mouse	Tu, 1974
(1969)	1.40	iv Mouse	Tu, 1974
(1972)	1.37	iv Mouse	Tu, 1974
	0.54	sc Mouse	Baxter & Gallichio, 1976
Year of collection (1967)	0.71	iv Mouse	Halstead et al., 1978

Table 1-4. Toxicities of Sea Snake Venoms and Components (Continued)

Venom or Component	LD50 (mg/Kg)	Route†/Animal	References
Year of collection (1964)	1.40	iv Mouse	Halstead et al., 1978
Lapemis Toxin	**0.06**	**iv Mouse**	**Tu & Hong., 1971**
	0.06	iv Mouse	Raymond & Tu, 1972
(20% iodination)	0.04	iv Mouse	Raymond & Tu, 1972
(42% iodination)	0.024	iv Mouse	Raymond & Tu, 1972
Laticauda colubrina			
Venom	0.42	sc Mouse	Tu et al., 1963
	0.45	sc Mouse	Levey, 1969
	0.40	iv Mouse	Sato et al., 1969
Laticauda laticoudata			
Venom	0.17	iv Mouse	Sato et al., 1969
	0.16	iv Mouse	Tu & Salafranca, 1974
	0.16	iv Mouse	Vick et al., 1975
Laticauda semifaciata			
Venom	0.211	iv Mouse	Tu, 1959
	0.063	iv Guinea pig	Tu, 1959
	0.048	iv Rabbit	Tu, 1959
	0.338	sc Mouse	Tu, 1959
	0.089	sc Guinea pig	Tu, 1959
	0.211	sc Rabbit	Tu, 1959
	0.05-0.06	Mouse	Tu & Passey, 1972
	0.325	sc Mouse	Baxter & Gallichio, 1976
	0.45	iv Mouse	Schmidt et al., 1976
	0.34	sc Mouse	Tu, 1977
	0.28	iv Mouse	Tu, 1977
	0.39	iv Mouse	Tu, 1977
	0.21	iv Mouse	Tu, 1977
	0.30	iv Mouse	Tu, 1977
	0.20	iv Mouse	Chang, 1979
	0.20-0.50	sc Mouse	Chang, 1979
	0.025	sc Rabbit	Chang, 1979
Laticauda semifaciata			
Toxin a	0.07	iv Mouse	Tu et al., 1971
Toxin b	0.05	iv Mouse	Tu et al., 1971
Erabutoxin a	0.15	im Mouse	Tamiya & Arai, 1966
Erabutoxin b	0.15	im Mouse	Tamiya & Arai, 1966
Toxin L5 III	1.20	iv Mouse	Karlsson, 1979

Table 1-4. Toxicities of Sea Snake Venoms and Components (Continued)

Venom or Component	LD50 (mg/Kg)	Route†/Animal	References
Pelamis platurus			
Venom	0.18	iv Mouse	Tu & Ganthavorn, 1969
	0.435	iv Mouse	Tu et al., 1975, 1976
	0.09-0.11	iv Mouse	Pickwell et al., 1974
	0.44	iv Mouse	Tu & Salafranca, 1974
	0.067	sc Mouse	Minton, 1983
Pure Toxin	0.044	iv Mouse	Tu & Salafranca, 1974
Pelamis Toxin a	0.044	iv Mouse	Tu et al., 1975
Pelamis Toxin b	0.15	iv Mouse	Tu et al., 1976
Pelamis Toxin c	0.31	iv Mouse	Tu et al., 1976

†(im - intramuscular; ip - intraperitoneal; iv - intravenous; sc - subcutaneous)

Table 1-5. Amino Acid Sequences of Sea Snake Neurotoxins.

#	Amino Acid Sequence
	10 20 30 40 50 60 70
1	MTCCNQQSSQPKTTTNCAGNSCYKKTWSDHRGTIIERGCGCPQVKSGIKLECCHTNECNN
2	MTCCNQQSSQPKTTTNCAGNSCYKKTWSDHRGTIIERGCGCPEVKSGIKLECCHTNECNN
3	LTCCNQQSSQPKTTTDCADNSCYKKTWQDHRGTRIERGCGCPQVKPGIKLECCKTNECNN
4	LTCCNQQSSQPKTTTDCADNSCYKMTWRDHRGTRIERGCGCPQVKPGIKLECCKTNECNN
5	LTCCNQQSSQPKTTTDCADNSCYKKTWKDHRGTRIERGCGCPQVKPGIKLECCKTNECNN
6	MTCCNQQSSQPKTTTNCAGNSCYKKTWSDHRGTIIERGCGCPQVKSGIKLECCHTNECNN
7	LSCYLGYKHSQTCPPGENVCFVKTWCDGFCNTRGERIIMGCAATCPTAKSGVHIACCSTDNCNIYAKWGS
8	LSCYLGYKHSQTCPPGENVCFVKTWCDAFCSTRGERIVMGCAATCPTAKSGVHIACCSTDNCNIYTKWGSGR
9	MTCCNQQSSQPKTTTNCAESSCYKKTWSDHRGTRIERGCGCPQVKPGIKLECCHTNECNN
10	MTCCNQQSSQPKTTTNCAESSCYKKTWSDHRGTRIERGCGCPQVKSGIKLECCHTNECNN
11	MTCCNQQSSQPKTTTNCAESSCYKKTWSDHRGTRIERGCGCPQVKKGIKLECCHTNECNN
12	MTCCNQQSSQPKTTTNCAESSCYKKTWSDHRGTRIERGCGCPQVKSGIKLECCHTNECNN
13	MTCCNQQSSQPKTTTNCAESSCYKKTWRDFRGTRIERGCGCPQVKPGIKLECCHTNECNN
14	MTCCNQQSSQPKTTTNCAESSCYKKTWSDHRGTRIERGCGCPQVKPGIKLECCHTNECNN
15	RICYLAPRDTQICAPGQEICYLKSWDDGTGFLKGNRLEFGCAATCPTVKPGIDIKCCSTDKCNPHPKLA
16	RICYLAPRDTQICAPGQEICYLKSWDDGTGSIRGNRLEFGCAATCPTVKRGIHIKCCSTDKCNPHPKLA
17	RRCYNQQSSQPKTTKSCPPGENSCYNKQWRDHRGSITERGCGCPKVKPGIKLRCCESEDCNN
18	RRCFNQQSSQPKTTKSCPPGENSCYNKQWRDHRGSITERGCGCPKVKPGIKLRCCESEDCNN
19	RRCYNQQSSQPKTTKSCPPGENSCYNKQWRDHRGSITERGCGCPTVKPGIKLRCCESEDCNN
20	RRCFNHPSSQPQTNKSCPPGENSCYNKQWRDHRGTITERGCGCPTVKPGIKLTCCQSDDCNN
21	RRCFNHPSSQPQTNKSCPPGENSCYNKQWRDHRGTIIERGCGCPQVKSGIKLTCCQSDDCNN
22	RRCFNHPSSQPQTNKSCPPGENSCYNKQWRDHRGTITERGCGCPTVKPGIKLTCCQSEDCNN
23	RRCFNHPSSQPQTNKSCPPGENSCYNKQWRDHRGTIIERGCGCPTVKPGIKLTCCQSEDCNN
24	RRCFNHPSSQPQTNKSCPPGENSCYNKQWRDHRGTITERGCGCPQVKSGIKLTCCQSDDCNN
25	RICFNHQSSQPQTT'TCSPGESSCYNKQWSDFRGTIIERGCGCPTVKPGIKLSCCESEVCNN
26	RICFNHQSSQPQTT 'CSPGESSCYHKQWSDFRGTIIERGCGCPTVKPGIKLSCCESEVCNN
27	RICFNHQSSQPQTT 'CSPGESSCYHKQWSDFRGTIIERGCGCPTVKPGINLSCCESEVCNN
28	RECYLNPHDTQTCE QEICYVKSWCNAWCSSRGKVLEFGCAATCPSVNTGTEIKCCSADKCNTYP
29	RICFNQHSSQPQTT 'CPSGQSSCYHKQWSDFRGTIIERGCGCPTVKPGIKLSCCESERCNN
30	MTCCNQQSSQPKTT. CAESSCYKKTWSDHRGTRIERGCGCPQVKSGIKLECCHTNECNN
31	MTCCNQQSSEPKTT CAESSCYKKTWSDHRGTRIERGCGCPQVKSGIKLECCHTNECNN

Table 1-5. Amino Acid Sequences of Sea Snake Neurotoxins (continued)

#	Species	Toxin Name	References
1	*Acalyptophis peronii*	Major Toxin	Mori & Tu, 1988a
2	*Acalyptophis peronii*	Minor Toxin	Mori & Tu, 1988b
3	*Aipysurus laevis*	Aipysurus laevis a	Maeda & Tamiya, 1976
4	*Aipysurus laevis*	Aipysurus laevis b	Maeda & Tamiya, 1976
5	*Aipysurus laevis*	Aipysurus laevis c	Maeda & Tamiya, 1976
6	*Astrotia stokesii*	Astrotia stokesii a	Maeda & Tamiya, 1978
7	*Astrotia stokesii*	Astrotia stokesii b	Maeda & Tamiya, 1978
8	*Astrotia stokesii*	Astrotia stokesii c	Maeda & Tamiya, 1978
9	*Enhydrina schistosa*	Es Toxin 4	Fryklund et al., 1972
10	*Enhydrina schistosa*	Es toxin 5	Fryklund et al., 1972
11	*Hydrophis cyanocinctus*	Hc Hydrophitoxin a	Liu & Blackwell, 1974
12	*Hydrophis cyanocinctus*	Hc Hydrophitoxin b	Liu & Blackwell, 1974
13	*Hydrophis lapemoides*	Hydrophis lapemoides a	Tamiya et al., 1983a
14	*Lapemis hardwickii*	Lapemis toxin	Fox et al., 1977
15	*Laticauda colubrina*	Laticauda colubrina a	Kim & Tamiya, 1982
16	*Laticauda colubrina*	Laticauda colubrina b	Kim & Tamiya, 1982
17	*Laticauda colubrina*	Laticauda colubrina c	Tamiya et al., 1983b
18	*Laticauda colubrina*	Laticauda colubrina d	Tamiya et al., 1983b
19	*Laticauda colubrina*	Laticauda colubrina II	Tamiya et al., 1983b
20	*Laticauda crockeri*	Laticauda crockeri a	Tamiya et al., 1983b
21	*Laticauda crockeri*	Laticauda crockeri b	Tamiya et al., 1983b
22	*Laticauda laticaudata*	Laticotoxin a	Maeda & Tamiya, 1976
23	*Laticauda laticaudata*	Laticauda laticaudata a	Tamiya et al., 1983b
24	*Laticauda laticaudata*	Laticauda laticaudata b	Tamiya et al., 1983b
25	*Laticauda semifasciata*	Erabutoxin a	Sato & Tamiya, 1971
26	*Laticauda semifasciata*	Erabutoxin b	Sato & Tamiya, 1971
27	*Laticauda semifasciata*	Erabutoxin c	Tamiya & Abe, 1972
28	*Laticauda semifasciata*	L. semifasciata III	Maeda & Tamiya, 1974
29	*Laticauda semifasciata*	Toxin b	Tsernoglou et al., 1977
30	*Pelamis platurus*	Pelamitoxin a	Wang et al., 1976
31	*Pelamis platurus*	Toxin b	Mori et al., 1989

Table 1-6. Conformation of Sea Snake Neurotoxins

Venom/Toxin	Method	Information	References
Enhydrina schistosa Major toxin	Raman	Mixture of turn and sheet with no helix	Yu et al., 1975; Tu, 1979
Lapemis hardwickii Lapemis toxin	Raman; CD	Mixture of turn and sheet with no helix	Yu et al., 1975; Fox & Tu, 1979
Laticauda semifasciata Toxin b	X-ray	Mixture of turn and sheet with no helix	Tsernoglou & Petsko, 1976, 1977
Laticauda semifasciata Erabutoxin a	CD; NMR	sheet and random coil	Inagaki et al., 1978
Laticauda semifasciata Erabutoxin b	X-ray;	Mixture of turn and	Miyazawa et al., 1983;
	Raman;	sheet with no helix	Inagaki et al., 1978;
	NMR; CD	His7 environment differs in solution from solid	Harada et al., 1976; Low et al., 1976; Kimball et al., 1979
Laticauda semifasciata Erabutoxin c	Raman	sheet and random coil	Takamatsu et al., 1980
Laticauda semifasciata LS III	NMR	Triple stranded anti-parallel pleated sheet	Inagaki et al., 1982
Pelamis platurus toxin a	Raman	Mixture of turn and sheet with no helix	Tu et al., 1976; Ishizaki et al., 1984

Table 1-7. Chemical Modification of Sea Snake Neurotoxins

Residue	Species	Toxin	Comment	Reference
Arginine				
	Laticauda semifasciata			
		toxin a:	No loss of toxicity when 1 of 3 residues modified	Tu et al., 1971
		toxin b:	No loss of toxicity when 1 of 2 residues modified	Tu et al., 1971
		LS III:	Half of toxicity lost	Kim et al., 1980
Disulfide bond				
	Pelamis platurus			
		Pelamis toxin:	Loss of toxicity	Tu et al., 1975
Histidine				
	Laticauda semifasciata			
		Erabutoxin b:	No loss of toxicity	Sato & Tamiya, 1970
Lysine				
	Laticauda semifasciata			
		toxin a:	No loss of toxicity when 3 of 4 residues modified	Tu et al., 1971
		toxin b:	No loss of toxicity when 4 of 5 residues modified	Tu et al., 1971
		LS III:	Loss of toxicity when Lys 23 or Lys 35 modified	Kim et al., 1980
Sulfhydryl group				
	Pelamis platurus			
		Pelamis toxin:	Less toxic but retains toxicity, still binds to AChR	Ishizaki et al., 1984
Tryptophan				
	Enhydrina schistosa			
		Major toxin:	Loss of toxicity	Tu & Toom, 1971
	Lapemis hardwickii			
		Lapemis toxin:	Loss of toxicity	Tu & Hong, 1971
			Loss of binding	Allen & Tu, 1985
	Laticauda semifasciata			
		Toxins a & b:	Loss of toxicity	Seto et al., 1970 Tu et al., 1971
Tyrosine				
	Lapemis hardwickii			
		Lapemis toxin:	Loss of toxicity	Raymond & Tu, 1972

Table 1-8. Toxicities of purified sea snake phospholipases A_2

Species and Toxin	Route	LD_{50} ($\mu g/g$)	Reference
	Subfamily Laticaudinae		
Laticauda colubrina			
Lc PLH-I	IV	>4.50	Takasaki et al. (1988)
Lc PLA-II	IV	0.05	Takasaki et al. (1988)
Laticauda semifasciata			
Ls PLA-I	IV	9.00	Takasaki et al. (1988)
Ls PLA-II	IV	>6.00	Takasaki et al. (1988)
Ls PLA-IV	IV	>6.00	Takasaki et al. (1988)
	Subfamily Hydrophiinae		
Enhydrina schistosa			
Toxin VI:5	IV	0.11	Fohlman & Eaker (1977)

Route refers to method of administration of toxin. IV = intravenous (tail vein). Venom toxicities are expressed in μg venom/g mouse body weight. Females of Swiss/Webster strains are typically used.

Table 1-9. Amino acid sequences of phospholipases A_2 from sea snake venoms

Species and toxin	10	20	30	40
Subfamily Laticaudinae				
Laticauda colubrina				
1. LcPLA-II	NLIQFSELIQ	CANKGKRATY	YYMDYGCYCG	KGGSGTPVDD
2. LcPLH-I	NLIQFSQLIQ	CANKGKRPTL	HYMDYGCYCG	PGGSGTPVDD
Laticauda laticauda				
3. L1PLA$_2$	NLAQFALVIK	CADKGKRPRW	HYMDYGCYCG	PGGSGTPVDE
Laticauda semifasciata				
4. LsPLA I	NLVQFSNLIQ	CNVKGSRASY	HYADYGCYCG	AGGSGTPVDE
5. LsPLA III	NLVQFTNLIQ	CANSGKRASY	HYADYGCYCG	AGGSGTPVDE
6. LsPLA IV	NLVQFSYLIQ	CANTGKRASY	HYADYGCYCG	AGGSGTPVDE
Subfamily Hydrophiinae				
Aipysurus laevis				
7. Al PLA$_2$	NLYQFDNMIQ	CANKGKRATW	HYMDYGCYCG	SGGSGTPVDA
Enhydrina schistosa				
8. PL	NLVQFSYVIT	CANHNRRSSL	DYADYGCYCG	AGGSGTPVDE

	50	60	70	80	90
1.	LDRCCKTHDD	CYGQAEKKGC	FPFLTLYNFI	CFPGGPTCDR	GTTCQRFVCD
2.	LDRCCKTNDD	CYGQAEKKGC	SPLSTNYNFD	CFPGGPQCGK	GTTCQRFVCD
3.	LDRCCKTHDQ	CYAQAEKKGC	YPKLTMYSYY	CDDGDPYCNS	KTECQRFVCD
4.	LDRCCKIHDN	CYGEAEKMGC	YPKWTLYTYD	CSTEEPNCST	KTGCQGFVCA
5.	LDRCCKIHDN	CYGEAEKMGC	YPKLTMYNYY	CGTQSPTCDD	KTGCQRYVCA
6.	LDRCCKIHDN	CYGQAEKMGC	YPKLTMYNYY	CGTQSPTCDN	KTGCQRYVCA
7.	LDRCCKAHDD	CYGVAEDNGC	YPKWTLYSWQ	CTENVPTCNS	ESGCQKSVCA
8.	LDRCCKIHDD	CYGEAEKQGC	YPKMLMYDYY	CGSNGPYCRN	VKKKCNRKVC

Table 1-9. (cont.)

	100	110	118	Reference
1.	CDIQAAFCFA	RSPYNNKNYN	INISKRCK	Takasaki et al. (1988)
2.	CDLKAALCFA	KSPYNNKNFN	IDTKKRCK	Takasaki et al. (1988)
3.	CDVRAADCFA	RYPYNNKNYN	INTSKRCK*	Guignery et al. (1987)
4.	CDLEAAKCFA	RSPYNNKNYN	IDTSKRCK	Nishida et al. (1982)
5.	CDLEAAKCFA	RSPYNNKNYN	IDTSKRCK	Nishida et al. (1982)
6.	CDLEAAKCFA	RSPYNNKNYN	IDTSKRCK	Nishida et al. (1982)
7.	CDATAAKCFA	EAPYNNKNYN	INTSNCQ*	Ducancel et al. (1989)
8.	DCDVAAAECF	ARNAYNNANY	NIDTKKRCK	Lind & Eaker (1981)

* This toxin sequence was deduced from the nucleotide sequence of cDNA.

Table 2-1 Hopp and Woods/ Levitt Hydrophilicity Values

Amino Acid	Hydrophilicity Value†	s values, (kcal/mol)*
Arginine	3.0	3.0
Aspartate	3.0	2.5
Glutamate	3.0	2.5
Lysine	3.0	3.0
Serine	0.3	0.3
Aspargine	0.2	0.2
Glutamine	0.2	0.2
Glycine	0.0	0.0
Proline	0.0	- 1.4
Threonine	- 0.4	- 0.4
Alanine	- 0.5	- 0.5
Histidine	- 0.5	- 0.5
Cysteine	- 1.0	- 1.0
Methionine	- 1.3	- 1.3
Valine	- 1.5	- 1.5
Isoleucine	- 1.8	- 1.8
Leucine	- 1.8	- 1.8
Tyrosine	- 2.3	- 2.3
Phenylalanine	- 2.5	- 2.5
Tryptophan	- 3.4	- 3.4

† Hydrophilicity values assigned by Hopp and Woods (1981).

* Solvent parameter values assigned by Levitt (1976).

The values of the hydrophobic solvent parameter s are taken as the measured free energy of transfer from water to ethanol in kcal/mol. The values range from 3.0 to - 3.4 with hydrophilic residues indicated by positive values and hydrophobic residues indicated by negative values.

Table 3-1 Sequences of Lapemis toxin and Its Synthetic Peptides

Lapemis toxin: (1-60) MW = 6,680

```
        10        20        30        40        50        60
        |         |         |         |         |         |
MTCCNQQSSQPKTTTNCAESSCYKKTWSDHRGTRIERGCGCPQVKPGIKLECCHTNECNN
```

Loop A_1: (3-17) (15 mer)

```
  3     10    17
  |     |     |
  CCNQQSSQPKTTTNC
```

Loop B_1: (22-39) (18 mer)

```
                22        30        39
                |         |         |
                CYKKTWSDHRGTRIERGC
```

Loop B_2: (23-38) (16 mer)

```
                23      30      38
                |       |       |
                YKKT  DHRGTRIERG
```

Loop C_1: (41-52) (12 mer)

```
                                      41      50 52
                                      |       |  |
                                      CPQVKPGIKLEC
```

Negative Control Nonsense Peptide (20 mer)

```
                    10        20
                    |         |
          EACDFGHIKLMNPQRSTVWY
```

Single Letter Designation Code of the Amino Acids

A - Alanine	G - Glycine	M - Methionine	S - Serine
C - Cysteine	H - Histidine	N - Asparagine	T - Threonine
D - Aspartic acid	I - Isoleucine	P - Proline	V - Valine
E - Glutamic acid	K - Lysine	Q - Glutamine	W - Tryptophan
F - Phenylalanine	L - Leucine	R - Arginine	Y - Tyrosine

Table 3-2 Sequences of Lapemis Toxin and Synthetic Peptides

Objective: To check the importance of the disulfide bond of the oxidized form of Peptide B1 as opposed to the reduced and alkylated form with respect to binding to the AChR since the results of binding studies indicated the Peptide B2 without the terminal cysteines did not appear to bind while the Peptide B1 in the oxidized from did bind with an apparent KD of 40 nM.

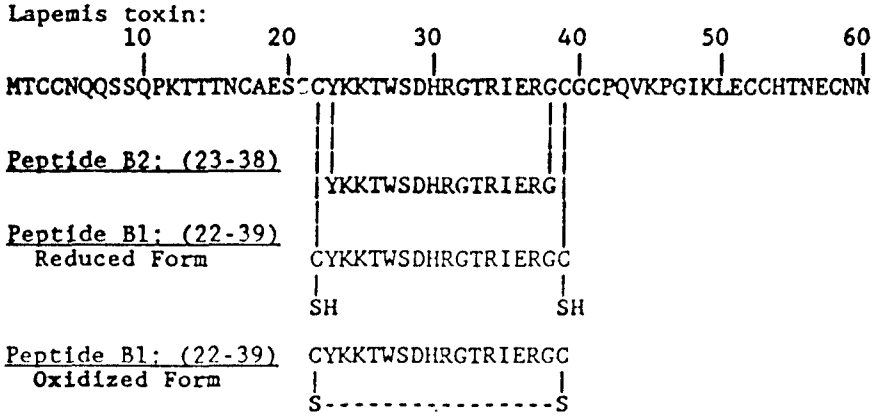

Table 3-3. Sequence Analysis of the Synthetic Peptides

Each peptide was attached to the Arylamine Membrane and applied to the MilliGen Sequencer (Model 6600). Amino acid residues found in each Edman degradation cycle [->] shown in the table using the single letter amino acid nomenclature. The peptides were not reduced and alkylated. The X indicates the lack of an amino acid in the Edman degradation cycle due to the non-alkylated cysteine residue at that postion.

Amount Spotted (pmol)	Amount Applied (pmol)	Synthetic Peptide Sample Name	Sequence Analysis Result Single Letter Sequence
200	60	Peptide A1:	X X N Q Q S S Q P K T T T N X ->->->->->->->->->->->->->->->
200	60	Peptide B1:	X Y K K T W S D H R G T R I E R G X ->->->->->->->->->->->->->->->->
200	60	Peptide B2:	Y K K T W S D H R G T R I E R G ->->->->->->->->->->->->->->->
500	140	Peptide C1:	X P Q V K P G I K L E X ->->->->->->->->->->->->
250	75	Nonsense	E A X D F G H I K L M N P Q R S T V W Y ->->->->->->->->->->->->->->->->->->

Average Percentage of Attachment (Initial Yield) was 29.6 %
Average Repetitive Yield was 94.8 %

Table 3-4 Amino Acid Analysis of Cobrotoxin.

Amino Acid	Integer (Res/molec)	Published (Res/molec)*
Asx	5	8
Glx	8	7
Ser	4	4
Gly	9	7
His	2	2
Arg	8	6
Thr	9	8
Ala	0	0
Pro	3	2
Tyr	3	2
Val	1	1
Met	0	0
Ile	3	2
Leu	1	1
Phe	0	0
Lys	4	3
Cys	n.d.	8
Trp	n.d.	1
Total Residues	60	62

Calculated MW 6750

Normalized on Val

* Yang, 1969

Table 3-5. Toxicity and K_D of the Synthetic Peptides and Neurotoxins

Sample	MW	K_D	LD50
-Bungarotoxin	7,976	1 nM	0.15 μg/g*
Lapemis toxin	6,680	2 nM	0.07 μg/g
Peptide A1	1,625	NBD	> 8 μg/g
Peptide B1	2,179	40 nM	> 8 μg/g
CA-Peptide B1	2,295	NBD	n.d.
Peptide B2	1,973	NBD	> 8 μg/g
Peptide C1	1,297	NBD	> 8 μg/g
Peptide NS	2,378	NBD	> 8 μg/g

* (Mebs et al., 1972)

n.d. - not determined NBD - No binding detected

Table 3-6 Amino acid composition of L. hardwickii PLA_2

Amino acid	Molar ratio†	Nearest integer*
Asx	2.54	20
Thr	0.67	5
Ser	0.33	3
Glx	1.27	10
Pro	0.52	4
Gly	1.20	10
Ala	1.00	8
EP-Cys	2.09	17
Val	0.26	2
Met	0.39	3
Ile	0.62	5
Leu	0.96	8
Tyr	1.34	11
Phe	0.37	3
His	0.43	3
Lys	0.77	6
Arg	0.39	3
Trp#	-	2
Total	15.15	123
Formula M_r	13999	
M_r (SDS-PAGE)	13500	

† Ratios normalized to alanine
* Based on an apparent MW of 13500 daltons
# Estimated by the method of Edelhoch (1967)

Table 3-7 N-terminus amino acid sequence of L. hardwickii PLA_2 and comparison with homologous PLA_2 from sea snake venoms

						5		
A.	Lapemis hardwickii PLA_2	N	L	Y	Q	F	K	N
B.	Laticauda semifasciata LsPLA I			V			S	
C.	Laticauda colubrina LcPLA-II			I			S	E
D.	Enhydrina schistosa PL			V			S	Y

			10					15					20			
A.	M	I	Q	C	A	N	H	G	S	R	M	T	L	D	Y	M
B.	L				N	V	K				A	S	Y	H		A
C.	L						K		K		A		Y	Y		
D.	V		T					N	R		S	S				A

		25					30					35				
A.	D	Y	G	C	Y	C	G	T	G	G	S	G	T	P	V	D
B.								A								
C.								K								
D.								A								

	40					45					50	
A.	E	L	D	R	C	C	K	I	H	D	D	- - - - - - -
B.											N	
C.	D							T				
D.												

References: A) Present work; B) Nishida et al., 1982; revised sequence: Takasaki et al., 1988; C) Takasaki et al., 1988; D) Lind and Eaker, 1981.

Blank spaces indicate residues identical with those of (A).

Table 3-8 Amino acid sequence of L. hardwickii PLA_2 8 kD fragment and comparison with homologous PLA_2 from sea snake venoms

	60							65							70						
A.	([M	-	L	-	C	-	G	-	P	-	Y	-	Y	-	N	-	L	-	Y	-	T	-	Y	-	D]-	C	-	V	-	E
B.		-	G	-		-	Y	-		-	K	-	W	-	T	-		-		-		-		-	S	-	T		
C.	K	-	G	-		-	F	-		-	F	-	L	-	T	-		-	N	-	F	-	I	-		-	F	-	P	
D.	Q	-	G	-		-	Y	-		-	K	-	M	-	L	-	M	-		-	D	-		-	Y	-		-	G	-	S

	75							80							85						
A.	H	-	Q	-	L	-	T	-	C	-	K	-	N	-	N	-	N	-	D	-	E	-	C	-	W	-	F	-	H	-	Q
B.	E	-	E	-	P	-	N	-		-	S	-	T	-	K	-	T	-	G	-	*	-		-	Q	-	G	-	F	-	V
C.	G	-	G	-	P	-		-		-	D	-	R	-	G	-	T	-	T	-	*	-		-	Q	-	R	-	F	-	V
D.	N	-	G	-	P	-	Y	-		-	R	-		-	V	-	K	-	K	-	K	-		-	N	-	R	-	K	-	V

	90				
A.	C	-	S	-	C -}
B.		-	A	-	-
C.		-	D	-	-
D.		-	D	-	-

References: A) Present work; B) Nishida et al., 1982; revised sequence: Takasaki et al., 1988; C) Takasaki et al., 1988; D) Lind and Eaker, 1981.

Blank spaces indicate residues identical with those of (A).
* Alignment space to allow for disulfide alignment
Methionine position in A inferred from sequence homology and first identified residue of 8 kD fragment
{ }-sequence obtained from Arg-C digest (see methods)
[ ]-sequence obtained from electroblot of 8 kD fragment

Table 3-9 Enzyme activity of native and metal-substituted Lapemis PLA_2 toward dipalmitoyl phosphatidylcholine

Treatment	μequivalents fatty acid released/min/mg protein	Fraction of Native Activity
None (Native)	66.57	1.00
EGTA	17.85	0.27
EGTA + Co^{2+}	2.10	0.03
EGTA + Ca^{2+}	59.85	0.90

PLA2 activity assayed by the titrimetric method of Wells and Hanahan (1969).
All assays run in replicate; values are minus controls.
See Methods for complete treatment procedures.

Table 3-10 Amino acid composition of L. hardwickii 9 kD Protein

Amino acid	MW	Molar ratio†	Nearest integer*
Asx	133	1.13	9
Thr	119	0.67	5
Ser	105	0.78	6
Glx	147	1.22	10
Pro	115	0.05	1
Gly	75	0.73	6
Ala	89	1.00	8
EP-Cys	121	0.23	2
Val	117	0.84	7
Met	149	0.25	2
Ile	131	0.54	4
Leu	131	0.90	7
Tyr	181	0.19	2
Phe	165	0.79	6
His	155	0.00	0
Lys	146	0.87	7
Arg	174	0.08	1
Trp#	204	-	-
Total		10.26	
Formula M_r			
M_r (SDS-PAGE)		8800	

† Ratios normalized to alanine
* Based on an apparent MW of 8800 daltons
# Not determined

Table 3-11 Amino acid sequence of tryptic peptides of 9 kD protein

Retention time (min)	Sequence
17.22	AGDTDG?GK
24.42	MLQAAESFSCK
27.30	SFFAK
28.94	VFEVIDQDK
29.39	TGYIEEGELK
31.84	MFMQNFDGK
39.93	IGVDEFVVLVSK

Retention times are those following RP-HPLC (Figure not shown).

Table 3-12 Amino acid sequence of L. hardwickii 9 kD protein and comparison with homologous boa constrictor parvalbumin

```
                                                                  5
A.  Lapemis hardwickii 9 kD protein     -  -  -  -  -
B.  Boa constrictor parvalbumin         A  F  A  G  I

                    10                  15                  20
A.  -  -  -  -  -  -  -  -  -  -  -  -  M (C) Q  A  A
B.  L  S  D  A  D  I  A  A  G  L  Q  S  C  Q  A  A
                 25                  30                  35

A.  E  S  F  S (C) K  S  F  F  A  K  -  -  -  -  -  -
B.  D  S  F  S  C  K  T  F  F  A  K  S  G  L  H  S
              40                  45                  50

A.  -  -  -  -  -  -  -  -  -  V  F  E  V  I  D  Q  D
B.  K  S  K  D  Q  L  T  K  V  F  G  V  I  D  R  D
     55                  60                  65

A.  K  T  G  Y  I  E  E  G  E  L  K  M  F  M  Q  N
B.  K  S  G  Y  I  E  E  D  E  L  K  K  F  L  Q  N
70                  75                  80                  85

A.  F  D  G  K  -  -  -  -  -  -  -  -  -  -  -  -  -
B.  F  D  G  K  A  R  D  L  T  D  K  E  T  A  E  F
                 90                  95                 100

A.  -  -  A  G  D  T  D  G  ?  G  K  I  G  V  D  E
B.  L  K  E  G  D  T  D  G  D  G  K  I  G  V  E  E
            105                 109

A.  F  V  V  L  V  S  K
                                    Blanks indicate
B.  F  V  V  L  V  T  K  G          no sequence available
```

Table 3-13 Amino Acid Composition of Gila Toxin

Amino Acid Residue	This Work min residues*	Hendon & Tu (1981) min residues*
Asx	2	3.9
Glx	3.4	4.3
Ser	3.9	4.2
Gly	5.6	6.2
Arg	1	1.07
Thr	2.0	2.0
Ala	2.6	2.48
Pro	2.4	2.5
Tyr	1.1	1.05
Val	3.2	3.02
Met	1	1
Ile	2.2	2.15
Leu	3.6	3.03
Phe	1	1
Lys	1.9	2.09
His	**	2.07
Trp	n.d.	***
Cm-Cys	n.d.	2.41

n.d. - not determined

* This Work min residue values normalized based on Phe as was done by Hendon & Tu.

** His not detected\ Analysis to be repeated

*** Trp was determined by Hendon & Tu using the spectrophotometric method of Edelhoch (1967) value 4.0 per 32,200 Mr protein.

Table 3-14 Carbohydrate Monosaccharide Composition of Gila Toxin

Monosaccharide	Molar content (nmol/nmol protein)
Fucose	0
Xylose	0
Manose	1.94
Galactose	1.74
Glucose	1.11
* N-Acetylgalactosamine	0
N-Acetylglucosamine	1.94
Salic acid	1.30
Total monosaccharide content	8.03

* The absence of N-Acetylglucosamine suggests a lack of any O-glycosylation of Gila Toxin

Table 3-15 Comparsion Gila Toxin* and G4

	Gila toxin	G4
	single polypeptide chain	single polypeptide chain
M.W.	35,000-37,500	33,000
pI	4.25	4
% Carbohydrate	8% (weight)	10% (weight)
Biological Assay activity	-No hemorrhagic activity -paralysis of the hind limbs	No hemorrhagic paralysis of the hind limbs

* Hendon and Tu Biochemistry 1981, 20, 3517-3522

Table 3-16 Enzymatic Activity of Gila toxin on Chromagenic Substrates

Substrate	Gila toxin
N-Benzoyl-Arginine Ethyl Ester (BAEE)	245 U/mg
N-Tosyl-Arginine Methyl Ester (TAME)	
N-Benzoyl-Phe-Val-Arg-pNA (Thrombin-like)	
N-Benzoyl-Ile-Glu-Gly-Arg-pNA (Factor X A)	0
N-Benzoyl-Val-Lue-Lys-pNA (Human plasmin)	0
N-Benzoyl-Val-Lue-Arg-pNA	0
Inhibited by DFP	Yes
Inhibited by EDTA	No

Table 3-17 Gila Toxin Sequence Comparison

Comparision of N-Terminal Amino Acid Sequences of Gila Toxin Crotalase Crotalus atrox (EI), Kallikrein-like and Thrombin-like Enzyme

	Residue no.	Reference
	5 10 15 20	
Gila Toxin	I I G G Q E C D E T G H P W L A L L H R S E	Thiswork
Crotalase	V I G G D K C N I N E H R F L V A L Y	Pirkle, 1981
Catrox (EI)	H V G G D E C N I N E H R S L V A I F	Bjarnason, 1983
Kallikrein	I I G G R E C E K N S H P W Q V A I Y F	Tschesche, 1979
Thrombin	I V E G S N A E I G M S P W Q V M L F	Butkowski, 1977

Table 4-1. Summary Synthetic Peptide Binding Studies to the Acetylcholine Receptor

Sample Name Sequence	K_D	Reference
Acetylcholine (normal ligand)	2.5 μM	Juillerat et al., 1982
Lapemis toxin MTCCNQQSSQPKTTTNCAESSCYKKTWSDHRGTRIERGCGCPQVKPGIKLECCHTNECNN	2.0 nM	this work
Peptide A1 CCNQQSSQPKTTTNC	NB	this work
Peptide B1 CYKKTWSDHRGTRIERGC	40 nM	this work
CA-Peptide B1 ca-CYKKTWSDHRGTRIERGC-ca	NB	this work
Peptide B2 YKKTWSDHRGTRIERG	NB	this work
Peptide C1 CPQVKPGIKLEC	NB	this work
-Bungarotoxin IVCHTTATIPSSAVTCPPGENLCYRKMWCDAFCSSRGKVVELGCAATCPSKKPYEEVTCCSTDKCNHPPKRQPG	1.0 nM	Schwendimann, 1984/this
Peptide LI/N IVCHTTATIPSSACTG	0.82 μM	Ruan et al., 1990
Peptide LI CHTTATIPSSACTG	NB	Ruan et al., 1990
Peptide LII CKMWADAFTSSRGKVVECG	0.15 μM	Ruan et al., 1990
Peptide LIII CPSKKPYEEVTCG	NB	Ruan et al., 1990
Peptide LIIIE ATCPSKKPYEEVTCG	0.44 μM	Ruan et al., 1990
Peptide LIVC CSTDKCNHPPKRQPG	NB	Ruan et al., 1990
Naja naja phil NTX LECHNQQSSQAPTTKTCSGETNCYKKWWSDHRGTIIERGCGCPKVKPGVKLNCCRTDRCNN	0.39 nM	Juillerat et al., 1982
Naja naja phil fragment TCSGETNCYKKWWSDHRGTIIERGCGCPKVKPG	0.22 μM	Juillerat et al., 1982

Underlined sequence letters indicate modifications or change from native toxin.

Table 4-2 Separation Time Limits on Detection of Binding

Relationship between equilibrium binding constant (K_D) and allowable separation time

K_D (M)	Allowable separation time (0.15 $t_{1/2}$)[a]
10^{-12}	1.2 days
10^{-11}	2.9 hr
10^{-10}	17 min
10^{-9}	1.7 min
10^{-8}	10 sec
10^{-7}	0.10 sec
10^{-6}	0.01 sec

[a]Calculations of $t_{1/2}$ (half-life for dissociation) assume an association rate constant of 10^6 $M^{-1} \times sec^{-1}$.

Courtesy of Dr. J. P. Bennet, Jr. (1978)

Table 4-3 Methods of Binding Studies

Separation procedures for receptor-ligand studies

Procedure	Separation time: Initial	+ Washing	= Total	Advantages	Disadvantages
I. Particulate preparations					
A. Filtration	0[a]	15 sec	15 sec	1. Speed and large sample capacity	1. Nonspecific ligand binding to filter materials
	5–15 sec[b]	15 sec	20–30 sec	2. Most thorough washing (lowest blanks)	2. Limitations as to amount of tissue which can be used without clogging filters
B. Centrifugation					
Macro scale	0[c]	5 sec[d]	5 sec	1. Rapid separation	1. Lower sample number
Micro scale	0[c]	0[e]	0	2. Small volume (micro scale)	2. Less efficient washing (higher blanks)
		10 sec[d]	10 sec		
II. Soluble preparations					
A. Dialysis	0	0	0	1. No separation time-dissociation artifact	1. Limited sample number 2. Nonspecific ligand binding to dialysis membrane
B. Adsorption of free ligand	Min	0	Min	1. Greater sample number than dialysis	1. Long separation time
C. Column chromatography	Min to hr	0	Min to hr	1. Effective for ligands not adsorbable	1. Very long separation time

[a] If incubation homogenate is not diluted before filtering.
[b] If incubation homogenate is diluted with buffer before filtering.
[c] No dilution of incubation homogenate before centrifugation.
[d] Rapid rinsing of pellet and tube.
[e] No washing or rinsing of pellet or tube.

Courtesy of Dr. J. P. Bennet, Jr. (1978)

Table 4-4 Artifact Detection in Binding Studies

Potential artifacts in receptor-binding studies

Source	Problem	Artifact(s)	Detection
I. Ligand	Radiochemical purity, specific activity	Overestimation of true ligand concentration with underestimation of binding constant or vice versa	1. Quantitative and qualitative analysis of radiochemical purity 2. Disparate binding parameters by saturation compared to displacement
	Self-association	Apparent negative cooperative behavior in dissociation studies (excess unlabeled ligand accelerates dissociation rate compared to infinite dilution); Such behavior may indicate the presence of true negative cooperative behavior as well	Use displacing agents (in dissociation studies) structurally different from ligand
	Association to antiadsorbent	Alteration of experimental K_D	Saturation studies with different concentrations of antiadsorbent, if utilized
	Different affinities of labeled vs. unlabeled ligand	Nonlinear Scatchard plots, apparent cooperative behavior	Comparison of Scatchard and Hill plots using labeled ligand alone and labeled ligand displaced by unlabeled ligand
II. Tissue	Nonreceptor saturable ligand binding	Apparent receptors	Structure activity studies
	Ligand metabolism	Binding of ligand metabolites, overestimation of free ligand concentration	Metabolism studies
	Tissue concentration too high (greater than 10% of added ligand bound)	Underestimation of binding constant	Knowledge of true free ligand concentration at equilibrium
III. Separation procedure	Separation procedure too slow for ligand dissociation rate	Loss of binding, underestimation of B_{max}, inaccurate determination of K_D	Comparison of K_D determined by equilibrium compared to kinetic studies
	Ligand binding to separation materials	Excessively high blanks, possible additional "receptor" populations	Ligand binding in absence of tissue

Courtesy of Dr. J. P. Bennet, Jr. (1978)

Section 7

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